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Evolution of parasite-host associations in *Spauligodon* nematodes: from the inside out

Maria de Fátima Esperança Jorge

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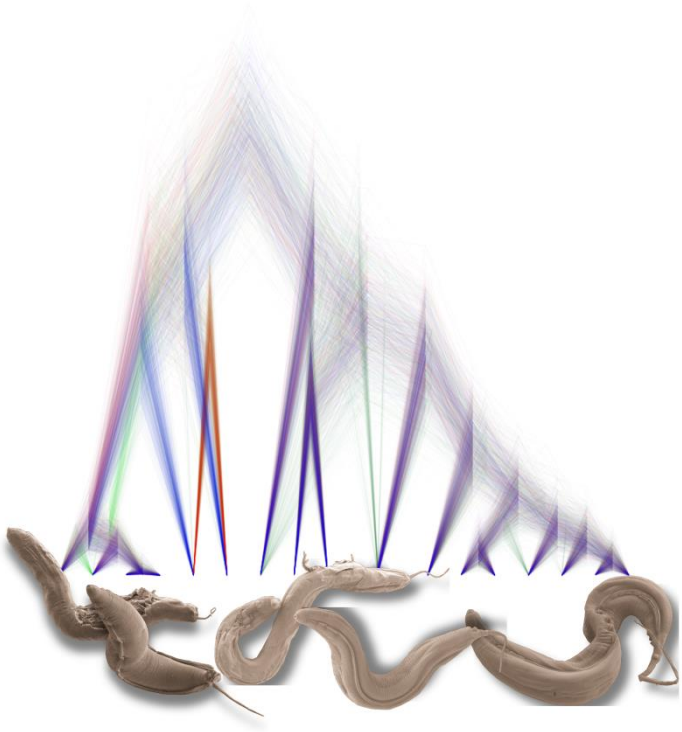

Evolution of parasite-host associations in *Spauligodon* nematodes: from the inside out

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Programa Doutoral em Biodiversidade, Genética e Evolução
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Nota Prévia

Na elaboração desta dissertação, e nos termos do número 2 do Artigo 4º do Regulamento Geral dos Terceiros Ciclos de Estudos da Universidade do Porto e do Artigo 31º do D.L. 74/2006, de 24 de Março, com a nova redação introduzida pelo D.L. 230/2009, de 14 de Setembro, foram incluídos em alguns capítulos, trabalhos de investigação já publicados ou submetidos em revistas internacionais indexadas e com arbitragem científica, formando parte de um conjunto coerente de trabalhos de investigação. Todos estes trabalhos são o resultado de colaborações, mas a candidata esclarece que, em todos eles participou na obtenção, interpretação, análise, discussão dos resultados e elaboração da sua forma publicada, sendo a primeira autora.

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"O valor das coisas não está no tempo que elas duram, mas na intensidade com que acontecem. Por isso, existem momentos inesquecíveis, coisas inexplicáveis e pessoas incomparáveis."

Fernando Pessoa

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Abstract

Interactions between organisms are one of the main drivers of biological diversification. At the core of these interactions are host-parasite associations. However, while the view of parasites as degenerate organisms is now changing, evolutionary biologists still do not fully recognize parasites potential as models of evolutionary processes. Only after we understand how parasites diversify and establish intimate associations with their hosts, can we assess how parasites fulfil such important roles in the ecosystem.

This thesis aimed to understand the processes driving host-parasite associations and parasite diversification, how these processes originate and are balanced, if these processes influence parasite evolutionary rates, and how parasite morphology has evolved. Parasitic nematodes belonging to the genus *Spauligodon,* were selected as a model system to explore these questions, which were addressed under an integrative evolutionary approach at molecular and morphological levels. Origins and evolution of host-parasite associations were studied in an island system, allowing a delimitation of the starting point of an oscillation period. The observed mosaic structure of *Spauligodon* diversity and host associations were explained by a combination of not “missing the boat” and association by descent; new host-parasite associations resulting from host-switches in early stages of post-colonisation, were explained on the basis of ecological fitting, later followed by association by descent boosted by host specificity. It was then evaluated the effect of parasite evolutionary history in the rate of molecular evolution, particularly codivergence versus host shifts. No significant differences in evolutionary rates between parasite lineages classified as congruent and incongruent were detected. The degree of incongruence between topologies and time, also appeared not to influence parasite evolutionary rate. The results from this new approach were ambiguous since it remains unclear if there is independence between rates and historical events in host-parasite coevolutionary associations, or such dependence only applies to a selected range of genes directly involved in host-parasite interaction, with no influence of demographic events.

After concentrating on the diversity of associations, and how they originated, diversified and constrained parasite molecular evolution, the focus of this thesis shifted to studying the morphological diversity of this parasitic taxon. In the studied parasitic system, we did not find any evidence that parasitism is correlated with a reduced morphological diversity. In fact, *Spauligodon* morphological diversity exhibits extreme phenotypic diversity with the existence of two alternative male morphs. The presence of male dimorphism in the *Spauligodon* taxon was interpreted as a result of alternative reproductive tactics. Additionally, no evidence of cryptic diversity (morphologically indistinguishable, but genetically distinct) was found. At least for male morphological characters, there is evidence of morphological differentiation between *Spauligodon* lineages. Morphological variation exhibited phylogenetic structure, and follows a Brownian model

of evolution, i.e. characters are evolving under neutral drift of character change with no selection, as oppose to non-divergent or convergent evolution.

Altogether, the studies included in this thesis provide insights of the evolutionary dynamic processes on the origin and evolution of host-parasite associations and parasite morphological diversification. A new approach to the study of rates of evolution in parasites was presented. To what degree the dynamic of host-parasite associations (i.e. host switch versus codivergence) affects the evolutionary rates and morphology is still to be determined. *Spauligodon* parasitic nematodes present diversity at several levels, including, host use, molecular and morphological levels. Hopefully, this thesis highlights the possibilities and value of parasites as evolutionary models.

Keywords

Alternative reproductive tactic, Canary Islands, codivergence, cryptic species, cytochrome oxidase subunit I, ecological fitting, evolution, host specificity, host switch, host-parasite associations, internal transcribed spacer 1, Macaronesian Islands, Mediterranean region, morphology, morphotypes, Nematoda, Oxyurida, parasite, parasite paradox, phylogeny, rate of molecular evolution, *Spauligodon*, species description, 18S ribosomal RNA, 28S ribosomal RNA.

Sumário

As interações entre os organismos são um dos principais impulsionadores da diversidade biológica. No centro dessas interações estão as associações parasita-hospedeiro. No entanto, ao mesmo tempo que a suposição de que os parasitas são organismos degenerados está a mudar, os evolucionistas ainda não reconhecem totalmente o potencial dos parasitas como modelos no estudo de processos evolutivos. Só depois de compreendermos como é que os parasitas se diversificam e conseguem estabelecer associações íntimas com os seus hospedeiros, é que poderemos avaliar de que maneira os parasitas conseguem executar funções tão importantes num ecossistema.

Esta tese teve como objetivo compreender os processos que levam às associações entre parasita e hospedeiro e à diversificação do parasita, como é que estes processos são originados e são equilibrados, se esses processos influenciam a taxa evolutiva do parasita, e como é que a morfologia do parasita evoluiu. Os nemátodos pertencentes ao género parasítico *Spauligodon* foram selecionados como um sistema modelo para explorar estas questões, que foram abordadas sob uma perspetiva evolutiva integrada a níveis moleculares e morfológicos. A origem e a evolução das associações parasita-hospedeiro foram estudadas num sistema insular, permitindo a delimitação de um ponto de oscilação de partida. A observada estrutura em mosaico da diversidade de *Spauligodon* e das associações com os hospedeiros foram explicadas por uma combinação de não "perder o barco" e de associação por descendência; novas associações parasita-hospedeiro resultantes de trocas de hospedeiro em períodos iniciais de pós-colonização foram explicadas com base num ajuste ecológico, seguido por uma associação por descendência impulsionada pela especificidade ao hospedeiro. De seguida, foi avaliado o efeito da história evolutiva do parasita na taxa de evolução molecular, particularmente co-divergência *versus* troca de hospedeiro. Não foram detetadas diferenças significativas nas taxas evolutivas entre linhagens do parasita, classificadas como congruentes e incongruentes. O grau de incongruência entre as topologias e o tempo também pareceu não influenciar a taxa evolutiva do parasita. Os resultados desta nova abordagem foram ambíguos, uma vez que ainda não está claro se há independência entre as taxas e acontecimentos históricos em associações co-evolutivas de parasita-hospedeiro, ou se essa dependência só se aplica a uma classe restrita de genes diretamente envolvidos na interação parasita-hospedeiro, sem a influência de eventos demográficas.

Após focalizar sobre a diversidade de associações, e como estas se originaram, diversificaram e limitam a evolução molecular do parasita, o centro desta tese deslocou-se para o estudo da diversidade morfológica deste grupo de parasitas. No sistema parasítico estudado, não foi encontrada qualquer evidência de que o parasitismo está relacionado com uma redução da diversidade morfológica. Na verdade, a diversidade morfológica do *Spauligodon* exibe diversidade fenotípica extrema com a existência de duas formas alternativas de machos. A presença de

dimorfismo em machos no grupo de *Spauligodon* foi interpretado como resultado de táticas alternativas de reprodução. Além disso, não foi encontrada nenhuma evidência para diversidade críptica (morfologicamente indistinguíveis, mas geneticamente distintos). Pelo menos para caracteres morfológicos de machos, há evidências para a diferenciação morfológica entre linhagens de *Spauligodon*. A variação morfológica exibiu estrutura filogenética e segue um modelo Browniano de evolução, ou seja, os caracteres estão a evoluir sob deriva neutra de mudança de caracteres sem nenhuma seleção, o oposto de evolução não divergente ou convergente.

No seu conjunto, os estudos incluídos nesta tese fornecem conhecimentos sobre os processos dinâmicos evolutivos sobre a origem e evolução das associações parasita-hospedeiro e da diversificação morfológica do parasita. Foi apresentada uma nova abordagem para o estudo das taxas de evolução nos parasitas. Até que ponto a dinâmica das associações parasita-hospedeiro (ou seja, troca de hospedeiro versus co-divergência) afeta as taxas evolutivas e a morfologia fica ainda por determinar. O nemátodo *Spauligodon* apresenta diversidade a vários níveis, incluindo o uso do hospedeiro, a níveis molecular e morfológicos. Esta tese destaca as possibilidades e o valor dos parasitas como modelos evolutivos.

Palavras-chave

Ajuste ecológico, associações parasita-hospedeiro, citocromo oxidase subunidade I, codivergência, descrição de espécies, espaço transcrito interno 1, espécies crípticas, especificidade ao hospedeiro, evolução, filogenia, Ilhas Canárias, Ilhas da Macaronésia, morfologia, morfotipos, Nematoda, Oxyurida, paradoxo do parasita, parasita, região Mediterrânica, *Spauligodon*, tática reprodutiva alternativa, taxa de evolução molecular, troca de hospedeiro, 18S RNA ribossomal, 28S RNA ribossomal.

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List of Abbreviations

12S: 12S ribosomal RNA
18S: 18S ribosomal RNA
1pW: Width of the 1st pair of caudal papillae
28S: 28S ribosomal RNA
2pL: Length of the 2nd pair of caudal papillae
2pW: Width of the 2nd pair of caudal papillae
3p1: Width of the papilla of the 3rd pair of caudal papillae at the tip
3p2: Width of the papilla of the 3rd pair of caudal papillae at the middle
3p3: Width of the papilla of the 3rd pair of caudal papillae at the insertion point
3pl: Length of the papilla of the 3rd pair of caudal papillae
3pW: Width of the 3rd pair of caudal papillae
AIC: Akaike Information Criterion
AICc: Akaike Information Criterion with a correction for finite sample sizes
ANCOVA: Analysis of covariance
ART: Alternative reproductive tactics
ASR: Ancestral state reconstruction
BI: Bayesian inference
BL: Body length
BM: Brownian model
bp: Base pairs
COI: Cytochrome oxidase subunit I
CT1: Caudal trunk width at its widest point
CT2: Caudal trunk width at its narrowest point
DNA: Deoxyribonucleic acid
EB: Early-burst model
ESS: Effective sample sizes
ExP: Excretory pore
HPD: Highest posterior density
invmtDNA: Invertebrate mitochondrial DNA
ITS1: Internal transcribed spacer 1
LA: Lateral alae
LDA: Linear Discriminant analysis
Legg: Egg length
Leggm: Average egg length
LS: Least square means

Ma: Mega-annum, a million years

MA: Larger male phenotype, or exaggerated morph

MANCOVA: Multivariate analysis of covariance

MANOVA: Multivariate analysis of variance

MCMC: Markov chain Monte Carlo

MI: Smaller male phenotype, or reduced morph

Mk1: Markov k-state 1

ML: Maximum likelihood

MP: Maximum parsimony

mrca: Most recent common ancestor

mtDNA: Mitochondrial DNA

Mya: Million years ago

NNet: Neighbor-Net

NR: Nerve ring

OBL: Oesophageal bulb length

OBW: Oesophagus bulb width

OL: Oesophagus length

OSR: Operational sex ratio

OU: Ornstein-Uhlenbeck model

OW: Oesophagus width

PC1: First principal component

PC2: Second principal component

PC3: Third principal component

PCA: Principal component analysis

PCR: Polymerase chain reaction

PextL: Posterior extremity length

PextW1: Width at the level of the 2nd pair of caudal papillae

PSRF: Potential scale reduction factors

RNA: Ribonucleic acid

SEM: Scanning electron micrographs

subs: Substitutions

TL: Tail length

TW: Tail width

Va: Vagina

Vu: Vulva

Wegg: Egg width

Weggm: Average egg width

"Para ser grande, sê inteiro.
Nada teu exagera ou exclui.
Sê todo em cada coisa.
Põe quanto és no mínimo que fazes.
Assim em cada lago a lua toda brilha, porque alta vive."

Fernando Pessoa

CHAPTER 1

General Introduction

Spauligodon aloisei, adult female

Parasitism is not just a lifestyle. Parasites establish intimate interactions with hosts, but not as passive unwelcome guests nor killing machines (while they can do it). Parasites are actually one of main drivers of diversity and are considered as ecosystems engineers (Hatcher et al. 2012) by structuring communities (Hatcher et al. 2014), being important components of food webs (Lafferty et al. 2008), affecting host morphology (Bordalo et al. 2014) and manipulating host behaviour (Poulin 2010). The limited view of parasites as evolutionary degenerate organisms is long gone. Parasites are complex resource users that offer a great model to study evolutionary process. What drives diversification? Such central evolutionary question is surely not less important than (and not complete unless): what drives parasites diversification? Knowledge of how parasites evolve and diversify both at the gene level but also at the phenotypic level is important for the understanding of some of the most fundamental aspects of evolutionary biology. This thesis presents an integrative evolutionary study of a parasitic nematode, genus *Spauligodon*, from genes to morphology (inside out), investigating how host-parasite association evolved, what are the consequences of different evolutionary events for the parasite and how morphology has evolved.

Parasites

What does it mean to be a parasite? Parasite is defined as “an organism living in or on another organism, the host—feeding on it, showing some degree of structural adaptation to it, and causing it harm” (Poulin 2007). Parasites do not represent a monophyletic group with a unique evolutionary transition from a free-living organism to a parasitic form. Such successful transition in lifestyle has evolved independently several times across several taxonomic groups, and accounts for close to half the species on Earth (Poulin and Morand 2000; Poulin 2011). Within taxonomic groups, some parasitic taxa appear to have evolved and diversified from a unique transition to parasitism of their ancestral form, for example in apicomplexan (Rayner and Keeling 2015), acanthocephalans (Herlyn et al. 2003), nematomorphs (Hanelt et al. 2005) among several others. Whether in nematodes (Blaxter et al. 1998), lice (Johnson et al. 2004), and many others, the transition to a parasitic lifestyle has occurred in several occasions. Such transition from a free-living organism to a parasitic one may represent the major evolutionary shift in life history strategy (Poulin and Randhawa 2015). Nonetheless, transition to a parasitic lifestyle is not irreversible (Siddal et al. 1993; Klimov and Oconnor 2013).

Apart from their taxonomic classification, parasites have been divided in categories. Following Anderson and May (1979) intensity-dependent mathematical models, parasites can be distinguished between microparasites (viruses, bacteria and protozoans) and macroparasites (parasitic helminths and arthropods). This dichotomy was more than a categorical classification scheme based on size, but also generation time, occurrence or not of reproduction within the host,

host immune response and nature of infection. Nevertheless, other traits are shared between both groups, as for example their life-cycle strategies. Recently, Poulin (2011) proposed a new classification of eukaryotic parasites according to six strategies: parasitoid, castrator, directly transmitted parasites, trophically transmitted parasites, vector transmitted parasites and micropredators (Fig.1.1). This classification highlighted the convergence to a limited set of adaptive peaks in the parasite evolutionary landscape, despite their wide phylogenetic diversity. Those strategies are identified based on the number of hosts used per parasite generation, the fitness reduction in the host, and the transmission routes used by the parasites (Poulin 2011). Convergent evolution in parasites is not restricted to their lifestyle nor to lifestyle strategies.

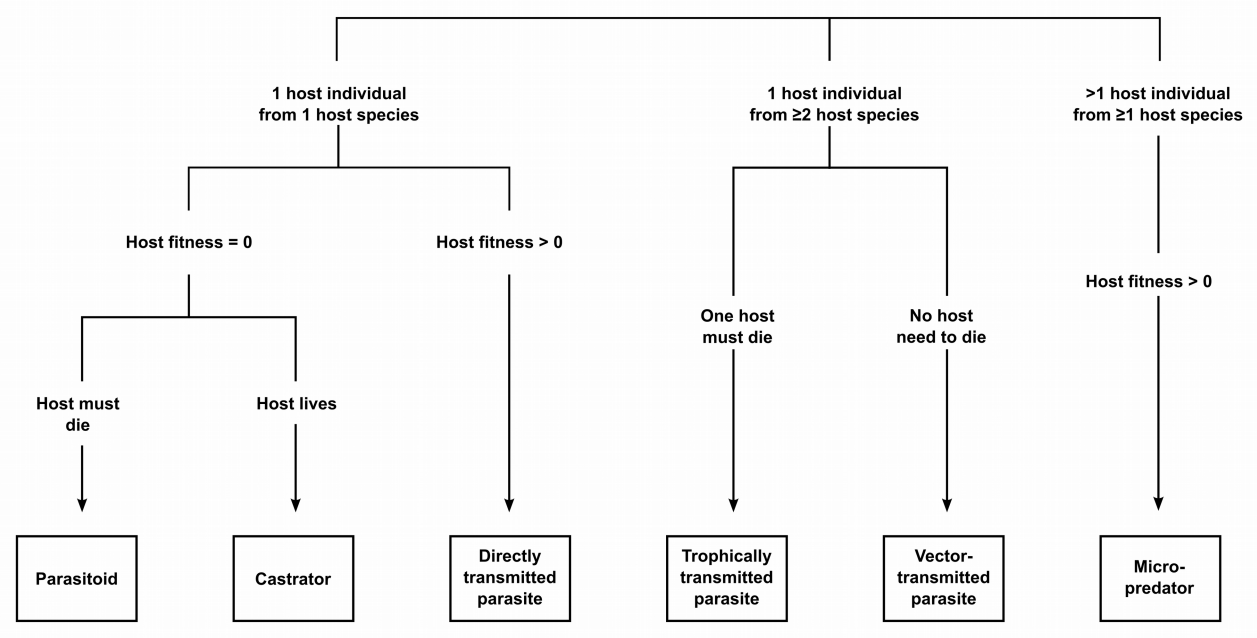

Fig.1.1- Classification tree of the six parasitic strategies considered by Poulin (2011). (adapted from Poulin 2011)

Convergence is also observed at morphological (Pérez-Losada et al. 2009), metabolic (Zarowiecki and Berriman 2015) and genomic levels (Poulin and Randhawa 2015). Along those convergence lines parasites seem to have evolved to an overall reduction in morphological traits, auxiliary metabolism and genome sizes. Such reduction may contribute to the idea that parasites represent degenerate organisms. However, parasites have also evolved new morphological adaptations (Hayunga 1991), genomic domains (Kikuchi et al. 2011) and unique solutions to common problems of immunity and secondary metabolism (Jackson 2015). Thus, parasites should not be considered organisms that have undergone degenerative evolutionary process, but rather highly specialized organisms which have evolved in a way to better survive and explore their host. The overall evolutionary convergence, as oppose to orthologous, at several different levels may provide the ultimate answer of what it takes to be a parasite in particular (Zarowiecki and Berriman 2015) and how evolution finds solutions to common problems.

Evolution of host-parasite associations

Parasites are defined by their dependency on host for habitat and other resources. What drives the evolution of host-parasite associations? Due to the intimate nature of host-parasite associations, parasites were expected to be host specific evolving in phylogenetic congruence with their host (Page 2003; Little et al. 2006; Dick and Patterson 2007). Phylogenetic congruence can be defined as the historical pattern produced by repeated cospeciation events (Clayton et al. 2003). In late 1800's, Fahrenholz defined a hypothesis of close correspondence between taxonomy of parasites and their hosts (Fahrenholz rule), leading to the hypothesis in cospeciation research that parasite phylogeny mirrors the one of the host. Several examples supported such hypothesis (Hafner and Nadler 1988; Page 2003). However, other macroevolutionary events also influence the structure of host-parasite associations resulting in incongruence (Page and Charleston 1998). Paterson and Banks (2001) acknowledged that parasite phylogeny may only imperfectly mirror host phylogeny and reformulated Fahrenholz' rule with their approach “through a glass, darkly”. Following this approach, the distribution of parasites would be a result of the interplay between several coevolutionary events: cospeciation, sorting, duplication and host switch events. This hypothesis still assumed cospeciation as a main determinant of the structure and history of host-parasite associations. Several other coevolutionary approaches shared the same assumption (Page 2003). However, current evidence actually suggests that phylogenetic congruence (as an approximation to cospeciation) between parasites and their hosts is not the rule but rather the exception (de Vienne et al. 2007; 2013). Host-parasite interactions usually do not lead to congruent demographic, phylogeographic or phylogenetic patterns (Nieberding et al. 2010). Indeed, coevolutionary dynamics of hosts and parasites do not favour long-term cospeciation (de Vienne et al. 2013). So what was wrong with that hypothesis? Incongruence was treated as “imperfections” because they failed to conform to the orthogenetic view of coevolution (Hoberg and Brooks 2008). Several coevolutionary events structure host-parasite associations, but incongruence is not a deviation from the main evolutionary road. The failure to find robust evidence for the initial null hypothesis that parasites mirror their host evolutionary trajectory was (sometimes still is) tangled with the assumption that “parasites are highly host-specific, hence they co-evolve with their hosts, and because they co-evolve with their hosts, they become highly host-specific” (Hoberg and Brooks 2008).

Host specificity is defined by the extent to which a parasite taxon is restricted in the number and relatedness of host species used at a given stage in its life cycle, which are constrained by both historical events and current ecological conditions (Poulin 2007). Host specificity has been correlated with phylogenetic signal strength, i.e. indicative of the suitability of hosts for a parasite being proportional to the phylogenetic distances between host species. (Presley et al. 2015). While parasites are often found to be quite specific to their host, what determines parasites specialisation

is likely to be complex, and parasites may be opportunistic in colonisation of new hosts (Poulin 2005). Indeed, observations of host shifts onto relatively unrelated hosts are common (Ricklefs et al. 2004; Brooks et al. 2006; Duval et al. 2007; Chapter 2).

The apparent contradiction of host use defines the Parasite Paradox (Agosta et al. 2010; Malcicka et al. 2015). How specialized parasites shift to new and sometimes unrelated hosts? The degree of specialisation of parasites does not necessarily follow a linear trend along their evolutionary history. Instead of representing an evolutionary dead end (Desdevises et al. 2002), it seems that specialisation is a two way road (Nosil and Mooers 2005; Poulin et al. 2006; Johnson *et al.* 2009). Agosta et al. (2010) proposed ecological fitting as the missing link to understand the evolution and diversification of host-parasite interactions. They adopted the initial idea from Janzen (1985) who observed that complex interactions established by introduced species may be only the consequences of a long succession of ecological fittings that do not require adaptations to evolve in new habitats; *"you don't have to be well-adapted to survive. You just have to survive*". Phenotypic plasticity, correlated trait evolution and phylogenetic conservatism, are the relevant mechanisms behind ecological fitting (Agosta and Klemens 2008). Ecological fitting provided a solution to the parasite paradox, in which specialised species may retain a phylogenetic capacity for host utilisation. Parasites can then specialise in the new host. Host shifts launched by ecological fitting are not the end-point, but rather a new starting point establishing novel associations (Agosta et al. 2010). The dynamics of persistence and diversification of host–parasite systems is indeed very complex, as observed in the dichotomy host-specificity – host shift. Host shifts seem to occur during periods of geographic expansion, while co-differentiation with hosts may occur during periods of geographic isolation or long periods of phenotypic stasis (Nieberding et al. 2008; Hoberg and Brooks 2008; 2010).

The study of parasite morphology

Morphology was the main source of information initially used in the identification of parasitic lineages. As noticed above, parasites have been classified as degenerate organisms. However, this view is often the result of an unbalanced comparison between parasites and their hosts (Poulin 2007). With the implementation of phylogenetics and now genomics, parasite identification and evolutionary study of parasitism have gained a new perspective (review in Littlewood 2013; Jackson 2015). Molecular data allow the proper formulation of phylogenetic hypotheses. Why do we need to study morphology? Morphology is part of organism diversity and is the product of several evolutionary processes that shaped organisms evolution. In the course of specialisation to their lifestyle, parasites underwent a different evolutionary trajectory from the one of their non-parasitic relatives. Certainly, some parasites have reduced morphological characters and organs

(Pérez-Losada et al. 2009; Haag et al. 2014; Zarowiecki and Berriman 2015). However, reduction or loss of organs and other morphological characters are also observed in cave animals (Protas and Jeffrery 2012). The question of how specialisation affects subsequent morphological evolution transcends parasitology (Svanbäck and Eklöv 2003; Gonzalez-Terrazas et al. 2012; Price et al. 2012). Parasites may reduce, but also evolve complex and diverse morphological structures in order to maximise resource use (Blaxter and Koutsovoulos 2015). Such morphological adaptation can be observed in intestinal helminths, with some having lost their own internal digestive systems completely, but they also present modifications of the tegument and development of specialized attachment organs (Hayunga 1991). Even in intracellular parasites such modifications have also occurred with the development of infection apparatus (Haag et al. 2014). Within groups, it is common to find morphologically indistinguishable, but genetically distinct parasites, i.e. cryptic species (Nadler and Pérez-Ponce de León 2011; Poulin 2011). Nevertheless, this is again documented in non-parasitic organisms (Adams et al. 2014). Morphological similarity may also be constrained by our (in)ability to identify reliable and diagnostic morphological characters. In many parasites, several morphological features are microscopic. High resolution tools are now commonly used, and when integrated with a detailed morphological analyses, they can uncover under-appreciated morphological diversity (De Ley et al. 2005; Jorge et al. 2013).

There are obvious challenges in the study of parasite morphology. Unicellular parasites represent one of the extremes of morphological variability. Even after detailed morphological studies, parasitologists may reach the conclusion that the species are indeed cryptic. There are only benefits in combining molecular and morphological data. An integrative evolutionary framework provides taxonomists and systematics with more tools to fulfil their main goal in describing species diversity (Padial et al. 2010; Razo-Mendivil et al. 2013). Integrating several sources of evidence, i.e. molecular and morphological data are not straightforward, and incongruence across the results may arise (Carstens et al. 2013, DeBiasse and Helberg 2015). But again, incongruence is still an important note of the evolutionary history of an organism. Congruent characters that underwent divergent evolution reflect groups phylogenetic relatedness suiting taxonomy proposes. Incongruence may result from processes leading to homoplasy, such as reversals, parallel evolution, convergence, as well as differential rates of change (Klingenberg and Gidaszewski 2010; Dávalos et al. 2012). For example, vulva appendages in nematodes demonstrate considerable homoplasy (Carta et al. 2009). Evolution of body size in female oxyurid parasitic nematodes is dependent on host body size, which can be a consequence of a longer lifetime and higher reproductive output (Morand et al. 1996). A better understanding of morphological evolution will improve our ability to utilise morphological characters in taxonomy and limit the level of incongruence with molecules, but also will contribute to the overall aim of understanding parasite evolution and diversification: inside out.

Parasites as evolutionary model system

It is not difficult to explain the importance of studying parasite evolution and diversification, especially when it comes to parasites as infectious agents. But as stated above, parasitism is so much more than the impact on their hosts. Parasitism represents a convergent evolutionary shift in life history strategy of several organisms. Several factors, such as the diversity of organisms that transitioned to a parasitic lifestyle, the diversity of host species used and their wide geographic distribution make parasites a great model system to study evolutionary process (Price 1980; Criscione et al. 2005; Poulin 2007). How are model organisms chosen? Accessibility and tractability are important traits, but the ultimate rationale depends on the ability to generalise to other organisms (Kellogg and Shaffer 1993). Models are the lenses through research problems are investigated, but can limit as much as facilitate the comprehension of process (Jenner and Wills 2007). One can wonder how we can generalise evolutionary patterns from parasites. Evolutionary biology has progressed from the studies on the nematode *Caenorhabditis elegans* (Kaletta and Hengarter 2006; Blaxter 2011) and the arthropod *Drosophila melanogaster* (Powell 1997; Beckingham et al. 2005). Within both nematode and arthropod groups there are several parasitic lineages. Diversity of organisms that adopt a parasitic lifestyle suggests a world of possibilities and different evolutionary questions that parasites can contribute to the answer, and certainly there are different models for different questions. While *C. elegans* and *D. melanogaster* have proven useful as a model organism in evolutionary studies, parasites do also offer tremendous potential as a model system for the study of evolutionary biology. For example:

Which are the processes shaping species diversification? Interactions among organisms are a fundamental driving force of species diversification (Thompson 2005). Parasitic organisms account for close to half the species on Earth (Poulin and Morand 2000), and as “ecosystems engineers” their influence goes from individual to ecosystem level (Hatcher et al. 2012). How can you ask such an important question without at least investing as much in answering how parasites diversify themselves (at genome, physiological and morphological level) and how they contribute to the diversification of other species?

Which are the subjacent factors influencing the variation in rates of molecular evolution across the tree of life? Body size, generation time and reproductive output have been correlated with the rate of evolution (Bromham 2002; Thomas et al. 2010). Parasites present higher rates of molecular evolution compared to their hosts (Haraguchi and Sasaki 1996) and non-parasitic relatives (Bromhan et al. 2013), which are expected to result from their life cycle peculiarities. To date, no comparative studies have been performed including several groups of parasites, and it is still unclear if the higher rates are a consequence of their lifestyle or demographic events. What other factors may influence the rate of evolution? The diversity of parasitic groups can once more prove useful. Is it their lifestyle, their short generation time, high reproductive out-put, size or evolutionary

events (i.e. frequency of host shifts) the factors that influence the evolutionary rate the most?

Parasitology, evolutionary ecology and evolutionary biology are just some of many disciplines. Understanding diversity, the process that drives diversity, interactions between species, lies in the intersection of all these fields. "[...] *ever since the dawn of civilization people have craved for an understanding of the underlying order of the world. There ought to be something very special about the boundary conditions of the universe. And what can be more special than that there is no boundary? And there should be no bound*ary [...]" (Stephen Hawking). There should be no boundaries in the study of diversification.

Study organism

The organism studied in this thesis is a taxon of parasitic nematodes, the genus *Spauligodon* Skrjabin, Schikhobalova et Lagodovskaja, 1960. It belongs to the Order Oxyurida (if following De Ley and Blaxter 2002: Order: Rhabditida, Infraorder: Oxyuridomorpha), Family Pharyngodonidade. Oxyurida nematodes (also commonly named pinworms) belongs to the Nematoda Clade III (*sensu* Blaxter et al. 1998: Ascaridida, Oxyurida, Spirurida and Rhigonematida; Fig.1.2). However, complete mitochondrial genome analysis has questioned the monophyly of this clade, and the position of Oxyurida within it (Park et al. 2011; Sultana et al. 2013).

Life cycle and transmission

Members of the order Oxyurida are microphagous nematodes inhabiting the posterior gut of various vertebrates and arthropods where they feed on the bacterial fauna (Anderson 1992). Among them, *Spauligodon* has only been found infecting reptiles. Oxyurida parasitic nematodes have a direct life cycle, requiring only one host to complete the life cycle (Fig.1.3). The host becomes infected from ingesting eggs containing third-stage larvae. Maturation and reproduction occur inside the host. Transmission occurs by deposition of eggs in aggregated groups or in some cases, gravid females also migrate to the cloaca of the host and function as ootheca (Adamson 1990). Since eggs dispersed into the environment are highly susceptible to desiccation, they present an overall low dispersal and transmission. The aggregated population structure of this parasite may favour transmission among related hosts, and in fact oxyuridian species are commonly restricted to individual host species (Adamson 1990). One additional peculiarity of this order is the presence of haplodiploidy as a form of reproduction (Adamson 1990, Adamson 1994). In haplodiploid organisms, males derive from non-fertilised eggs and are haploid, whereas females derive from fertilised eggs and are diploid. A direct consequence of this for transmission is that only an isolated female is needed to colonise a new host. Yet, another peculiarity occurs within

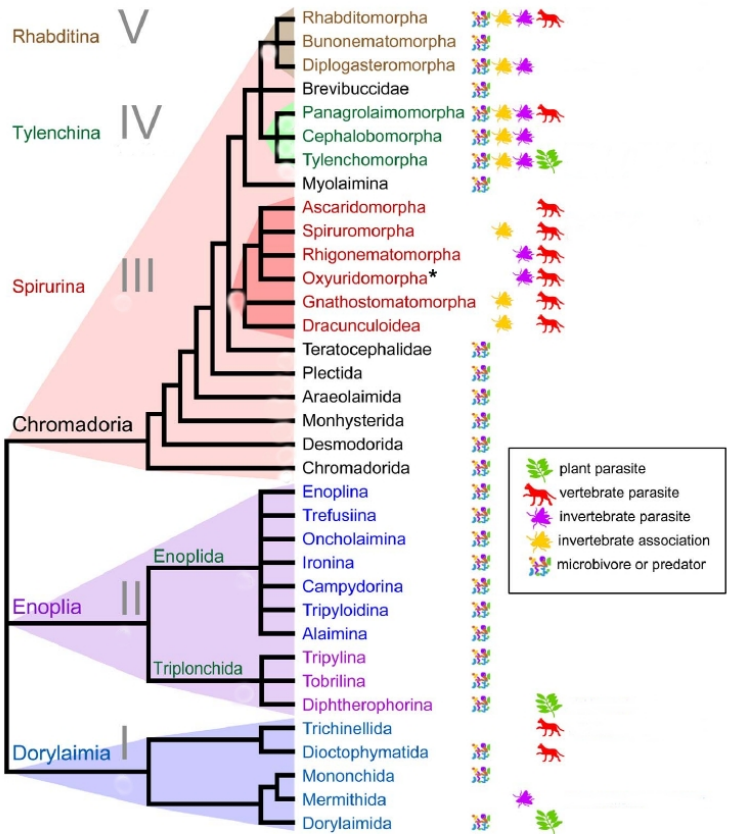

Fig.1.2- Nematoda inferred phylogeny based on molecular phylogenetic analyses with the small subunit ribosomal RNA gene. The systematic names given by De Ley and Blaxter (2002) are given, as is the ‘‘clade’’ naming convention introduced by Blaxter et al. (1998). Feeding mode, and animal and plant parasitic and vector associations, are indicated by small icons. Group to which *Spauligodon* belongs is indicated by an asterisk. (adapted from Blaxter 2011)

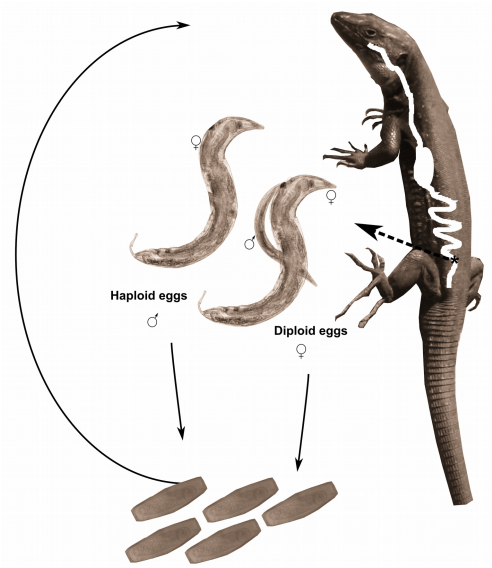

Fig.1.3- Life cycle of *Spauligodon* parasitic nematode.

Pharyngodonidade family namely in *Skrjabinodon* and *Spauligodon*, where male dimorphism has been reported (Ainsworth 1990; Jorge et al. 2014). However it remains unclear if it represents an

alternative reproductive tactics (Jorge et al. 2014).

Morphology

Like other oxyurids, *Spauligodon* nematodes have a straight oesophagus that ends in subspherical bulb. They are sexual dimorphic, females being bigger than males (Fig.1.4). Members of the genus *Spauligodon* are identifiable by the caudal alae in males not being supported by the postcloacal papillae pair. Vulva opening is located in the first half of the female body. Species determination is based on the structure of the male caudal extremity, since females seem to be very similar between groups. Being related with reproduction, the caudal extremity of the male of the Pharyngodonidae also seems to better show the phylogenetic relationships of the group (Anderson et al. 2009).

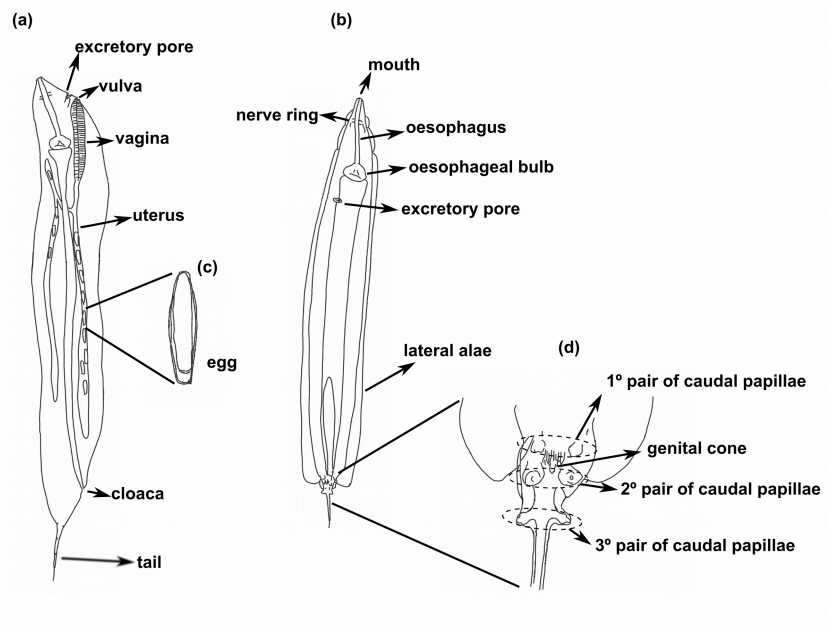

Fig.1.4- Schematic representation of the general anatomy of *Spauligodon* adult female (a) and male (b). (c) detail of an egg. (d) detail of male posterior extremity. (Note: position of excretory pore and vulva in females vary between different species.)

Spauligodon parasitic nematodes represent a great model to study evolutionary process shaping host-parasite associations, as well as morphology. Several traits described above, namely, life history, aggregated population structure, dispersal limitations, together with the low dispersal of their hosts and the observation of host-switching events along *Spauligodon* evolutionary history (Chapter 2 and 3) make this parasite particularly valuable in gaining a better understanding of the process leading to diversification in parasites in particular, but also in living organisms in general.

Thesis objectives and structure

This thesis provides an integrative evolutionary study of the parasitic nematode, genus

Spauligodon. Parasite evolutionary history was assessed at molecular and morphological levels (from the inside out). The main questions addressed in this thesis were:

How parasites evolve as part of a host-parasite system?:

How host parasite interactions are established and evolve?

Which is the balance between host specificity and ecological fitting?

Which are the evolutionary consequences for parasites of host codivergence versus host shift?:

Will it alter the rate of molecular evolution?

Is there limited morphological diversity in parasites?:

What drives the existence of alternative male phenotypes?

Is there any cryptic diversity?

Can morphology be used to discriminate between parasite lineages?

This thesis is organised in seven chapters and followed by appendixes. Chapter 1, the current chapter, provides a general introduction to the subjects and questions developed in the following chapters.

Chapter 2 presents a study of the origins and evolution of host-parasite associations in Canary Islands. The main aim was to investigate how host parasite associations are established and evolve. It focuses on parasite specificity – ecological fitting dynamics that shape the evolutionary history of host-parasite interactions. This study is a pre-submission version.

Chapter 3 follows the observation from the previous Chapter 2 that host switches occurred along the evolutionary history of *Spauligodon* parasitic nematodes. It focuses on the effect of parasite evolutionary history on the rate of molecular evolution, with expectations that different evolutionary events would have an effect at the whole genome-level. The study tested the hypothesis that parasite lineages evolving in congruence with host phylogeny present an overall slower evolutionary rate, in contrast to those resulting from switches to new, unrelated hosts which will have relatively higher rates. This study is a pre-submission version.

Chapter 4 is a study on the evolution of alternative male morphotypes. Genetic and ecological data were combined to investigate the presence of male dimorphism across several *Spauligodon* species. Specifically, two hypotheses were tested: (1) similar patterns of male dimorphism have evolved convergently within different species; and (2) the occurrence of the minor male morphs is frequency-dependent in natural infections as a possible consequence of alternative reproductive tactics. This study was published in the *Journal of Evolutionary Biology.*

Chapter 5 presents a formal species description. A detailed morphological study was

conducted to determine putative phenotypic differences between two *Spauligodon* lineages previously identified in a molecular study and considered as cryptic. This study was published in the *Journal of Zoological Systematics and Evolutionary Research*.

Chapter 6 provides a combined framework to investigate the reliability of male morphological characters as a diagnostic tool for lineage discrimination within the genus *Spauligodon*. A phylogenetic comparative method was used to assess how morphological characters reflected the relatedness between lineages, and how they have evolved. This study is also in a pre-submission version.

Chapter 7 consists of a general discussion summarising the main findings of this thesis and setting new research questions to be followed up in the future as a direct result of the thesis findings.

Appendix A provides a comparative methodological study in differences in detectability of intestinal parasites in reptiles using the classical invasive approach (intestine dissection), versus a non-invasive procedure (faecal examination). This study was published in *Parasitology Research*.

Appendix B, C, D and E provides all supplementary materials from Chapters 2, 4, 5 and 6, respectively.

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CHAPTER **2**

Why no simple answers? Reconstructing the complex colonisation history of the parasite *Spauligodon* (Nematoda) in the Canary Islands.

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Spauligodon atlanticus, adult male

Abstract

The Parasite Paradox refers to the apparent inconsistency in the fact that even as resource specialists, parasites often shift onto relatively unrelated host species, a phenomenon which might be explained by ecological fitting. Oceanic islands represent an ideal system in which to study parasite diversification under such duality. In this study, we infer the origin and evolution of host-parasite associations of a taxon of nematodes parasitic in reptiles, the genus *Spauligodon*, in the Canary Islands. Parasite phylogeny and divergence time estimates were inferred from molecular data with Bayesian methods. We then tested for evolutionary congruence for the most common clade, with a global-fit method. The results suggest the presence of four *Spauligodon* clades, which originated from at least three different colonisation events. Within two clades, lineage diversification originated from a host switching event between skinks and lacertid lizards, followed by separate evolution. Overall, while island colonisations by parasites are initially determined by stochastic events (whether or not they 'get in the boat'), the initial colonisation stage represents an oscillation period during which the parasite diversification can be mostly explained by ecological fitting, followed by a period of host specialisation.

Introduction

Islands, the so called "natural laboratories" (Mayr 1967), have been the preferred study setting of many evolutionary studies (Emerson 2002; Helmus et al. 2014; Kueffer et al. 2014; Warren et al. 2015). Their geographic isolation and restricted scale, presenting well delimited physical barriers to gene flow, shape unique selective pressures acting on their colonisers. In island systems, each new emerged island represents a new empty habitat, functioning as a replicate where each evolutionary pattern can be distinguished from unique outcomes (Losos and Ricklefs 2009). Therefore, insular organisms may represent the best models to understand the evolutionary processes shaping species diversification. The interest on the geographical and evolutionary dynamics of island biota have generally focussed on plants and vertebrates (Kueffer and Fernández-Palacios 2010; Sanmartín et al. 2008; Algar and Losos 2011). However, those new island colonisers rarely travel alone; these taxa are themselves habitat for other organisms, such as parasites, rafting on them during colonisation. But how have existing host parasite interactions evolved on islands?

Parasitism is one of the most common lifestyles (Poulin 2014). Due to the characteristics and requisites of their lifestyle, parasites are expected to be host specific (Page 2003; Little et al. 2006; Dick and Patterson 2007). Nevertheless, host shifts onto relatively unrelated hosts are common (Ricklefs et al. 2004; Brooks et al. 2006; Duval et al. 2007). Such contradiction of host use defines the Parasite Paradox (Agosta et al. 2010; Malcicka et al. 2015). Host specificity is defined by the extent to which a parasite taxon is restricted in the number and relatedness of host species used at

a given stage in its life cycle, which are constrained by both historical events and current ecological conditions (Poulin 2007). However, the degree of specialisation of parasites does not necessarily follow a linear trend along their evolutionary history. Instead of representing an evolutionary dead end (Desdevises et al. 2002), it seems that specialisation is a two way road (Nosil and Mooers 2005; Poulin et al. 2006; Johnson et al. 2009), in which specialised species may retain the potential for ecological fitting during oscillations in host range, explaining the origin of novel associations (Agosta et al. 2010). In this context, island systems may represent one of the best contexts to study the origin and evolution of host-parasite associations, with new colonisations defining the starting point of an oscillation period.

The distribution of parasites is superimposed (but not mirrored) on that of their host, and their evolutionary trajectories are shaped in a different way than that of non-parasitic organisms, since, generally, parasites colonise the islands in the “comfort” of their known habitat, the host. However, as for their counterpart, the conditions of transmission may be compromised. Similarly to non-parasitic taxa, several factors may favour the colonisation of some parasites over others, such as the complexity of the life cycle (i.e. the number of obligatory hosts), their life strategies (i.e. free living stages, formation of cysts), prevalence and intensity. In this context, parasites with direct life cycles have higher chances of successfully colonising new habitats such as islands if their hosts also succeed. Post-colonisation, several events may also shape their evolutionary history. Previous studies in islands have shown that parasites do suffer from similar founder effects as their hosts: impoverishment of species richness in islands (Roca et. al. 2009), loss of genetic diversity and additionally higher host-switching and lower specificity compared to their mainland relatives (Nieberding et al. 2006; Pérez-Rodríguez et al. 2013). This last phenomenon could be the direct result of reduced specialization, a possible evolutionary adaptation to islands in particular, or to hosts shifts in the general context of ecological fitting by resource-tracking (Agosta et al. 2010; Malcicka et al. 2015). Studies on blood parasites inhabiting islands suggest that diversity seems to be explained by a combination of two main processes, multiple immigrations and post-colonisation adaptations to specific hosts (Cornuault et al. 2012; Gómez-Díaz et al. 2012). However, depending on the degree of host specificity, host phylogeography can be the most important determinant of parasite phylogeographic structure (Nieberding et al. 2008; Falk and Perkins 2013). Therefore, what is the balance between host specificity and ecological fitting in islands? Do both phenomena play different roles at different stages in the dynamics of host-parasite evolutionary history in insular systems?

To answer these questions, we studied the origins and evolution of host-parasite associations involving a taxon of obligate parasitic nematodes in reptiles of the Canary Islands. The Canary Islands are one of the best studied models of oceanic archipelagos (Juan et al. 2000; Dietzen et al. 2008; Sanmartín et al. 2008; Husemann et al. 2014), located approximately 100 Km from the

northwestern coast of Africa and forming a chain of seven main islands and several islets. All islands were formed during the past 20 Mya, in an east-to-west formation sequence by a volcanic hotspot in the Atlantic Ocean (Fig.2.1a and b; Guillou et al. 2004; Ancochea et al. 2006; Sanmartín et al. 2008). While the majority of the islands arose from a single edifice, the current Tenerife Island resulted from the union of three independent and consecutive shield volcanoes (Guillou et al. 2004). Many of the Canarian endemic taxa appear as non-monophyletic, mostly due to multiple independent colonisation events associated with the ancient origin of the islands and close proximity to the mainland (Juan et al. 2000; Sanmartín et al. 2008). But despite the proximity to the continent, dispersal from Africa to the Canary Islands may not be very important when explaining the diversification patterns within lineages due to the dominant northern marine currents (Sanmartín et al. 2008). In fact, dispersal to or from other nearby archipelagos (Cape Verde, Selvagens and Madeira) is also observed (Carranza et al. 2002; Kim et al. 2008). Among vertebrates, reptiles are one of the most interesting groups, with 13 native species grouped into three genera (*Tarentola* geckos, *Chalcides* skinks and *Gallotia* lizards), one of which (*Gallotia*) is endemic to this archipelago. Interestingly, each taxon has colonised the archipelago following a distinct route (Carranza et al. 2002, 2008; Cox et al. 2010) (Fig.2.1c, d and e), but only *Gallotia* lizards appear to be the result of a single colonisation event (Cox et al. 2010). One of the common reptile parasitic nematodes present on the Canary Islands belongs to the genus *Spauligodon*. This nematode is an obligate parasite with a direct oral-faecal life cycle and no free-living stages, that infects the reptiles' intestine (Adamson 1990). Transmission occurs with nematode eggs being deposited together with host faeces in an aggregated pattern, which together with a high susceptibility to desiccation results in a low dispersal ability (Adamson 1990).

This system is thus especially well-suited to study parasite specificity – ecological fitting dynamics shaping the evolutionary history of host-parasite interactions, due to: (1) the islands history and age; (2) the low dispersal ability of the hosts, as well as the different routes of historical colonisation of the archipelago by the three host genera; and (3) parasite life history traits and dispersal limitations. To uncover the evolutionary dynamics of diversification of this parasite taxon in the Canary Islands, we extended a previous study on *Gallotia* lizards (Jorge et al. 2011) to include all other potential endemic reptile hosts, namely *Tarentola* geckos and *Chalcides* skinks. We first estimate the phylogeny of the parasites to assess the number of monophyletic groups that may represent independent colonisation events and determine parasite specificity by identifying the contemporaneous parasite-host associations. Then, we use an estimation of the time calibration phylogenetic trees to infer when they originated, as well as the ancestral geographical and host ranges. Finally, we determine the possible evolutionary scenarios responsible for the observed parasite diversity patterns by estimating the degree of congruence between parasite and host phylogenies.

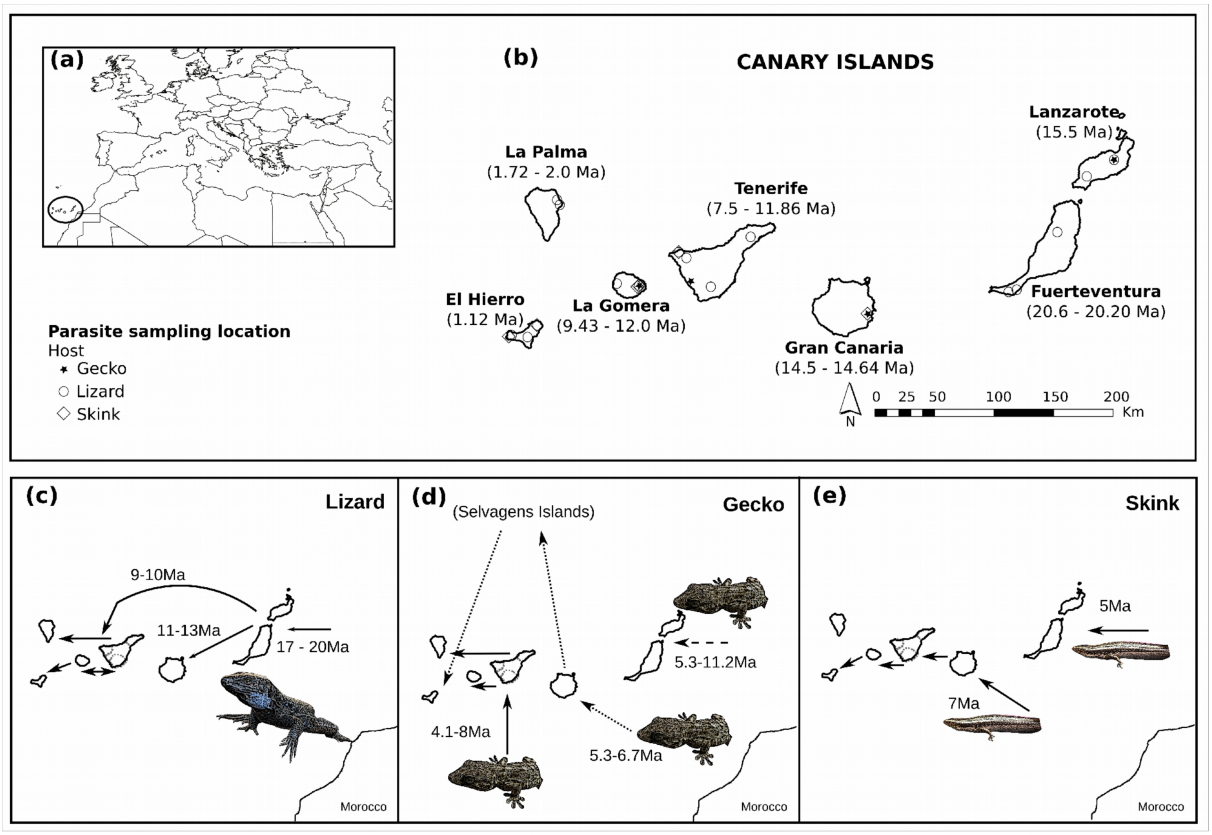

Fig.2.1- a) Geographical location of the Canary archipelago; b) Canary Islands with the approximate ages of the islands (Carracedo et al. 1998; Guillou et al. 2004); Main colonisation routes with estimated ages for: c) the *Gallotia* lizards (Cox et al. 2010), d) *Tarentola* geckos (Carranza et al. 2002) and e) *Chalcides* skinks (Carranza et al. 2008).

Material and Methods

Taxon and character sampling

A total of 292 *Gallotia* spp. lacertid lizards (thereafter lizard), 151 *Chalcides* spp. skinks (thereafter skink) and 360 *Tarentola* spp. geckos (thereafter gecko) were sampled in the Canary Islands between 2012 and 2014, in an effort to cover all different non-threatened species and subspecies of the three potential host genera. Samples consisted of faecal pellets (612, including more than one pellet per host in some cases) and intestine contents (227). Additional faecal samples were also collected from several reptile groups from different geographical areas (see Data B2.1, Supporting Information). Samples were collected and processed as described in Jorge et al. [2014 (Chapter 4)]. From the total of 803 sampled hosts, only 163 were found to be infected with *Spauligodon* parasites. From these, representatives from all infected host species and localities available for the Canary Islands, as well as from several *Spauligodon* species from other regions (Data B2.1, Supporting Information) were selected for molecular analyses to provide a more robust framework for phylogenetic inference. Extraction of genomic DNA was performed on individual nematodes using the PureLink® Genomic DNA Kit (Invitrogen, Invitrogen New Zealand

Ltd, Auckland, New Zealand) according to the manufacturer's protocol. We included three gene fragments in our analyses: two nuclear genes, the 18S ribosomal RNA (18S) and 28S ribosomal RNA (28S) and one mitochondrial gene, the cytochrome oxidase subunit I (COI). DNA extraction and polymerase chain reactions (PCR) were performed as described in Jorge et al. [2014 (Chapter 4)].

Samples from all hosts were also collected from tail tips and stored in 96% ethanol for genetic confirmation of species identity, if needed, and for posterior cophylogenetic analysis. Total genomic DNA was extracted from small pieces of tail using standard methods (Harris et al. 1998). A partial gene fragment of the mitochondrial 12S rRNA (12S) was amplified and sequenced using the primers 12Sa and 12Sb (Kocher et al. 1989). The amplification process was performed as described in Harris et al. (1998). PCR products purification and sequencing was performed by a commercial facility (Macrogen Corporation, http://www.macrogen.com).

Molecular data

A total 48 new nematode specimens were successfully amplified, 31 of them representing new haplotypes in the Canary Islands. Sequence chromatograms of *Spauligodon* were edited, trimmed and exported as nexus files in GENEIOUS v8.1.4 (http://www.geneious.com, Kearse et al. 2012). Thirty-eight additional *Spauligodon* sequences from the Canary Islands and other geographical localities published in previous studies were also included (Data B2.1, Supporting Information) in the parasite dataset. *Parapharyngodon echinatus* and *Thelandros tinerfensis* were used as outgroups (Data B2.1, Supporting Information). The dataset for each DNA fragment was aligned using MAFFT (Katoh et al. 2002), in CIPRES Science Gateway v3.3 (Miller et al. 2010), employing the G-INS-i and Q-INS-i algorithms (default parameters) for protein-coding and ribosomal regions, respectively. Three relevant specimens lacked sequence information for some of the genes (two for 28S and one with a shorter 79bp amplicon for COI). However, given the importance of those samples and because missing data is expected to have minor impact on the accuracy of phylogenetic analysis and divergence dating (Wiens and Morril 2011; Zheng and Wiens 2015), these sequences were included in the analyses but coded as "?". To correct possible mistakes and to determine the reading frame, COI alignment was visually inspected and translated to amino acids, specifying invmtDNA gene code, in GENEIOUS v8.1.4 (http://www.geneious.com, Kearse et al. 2012). Possible substitution saturation in the codon partitions was evaluated by implementing the Xia test (Xia et al. 2003; Xia and Lemey 2009) and by plotting genetic distances (uncorrected p-distances and JC69 model) against the number of transitions and transversions, as implemented in DAMBE v5.3.48 (Xia 2013). The aligment of the host 12S sequences corresponding to each host-parasite link of the parasite dataset was performed as described above for the nuclear parasite markers.

Phylogenetic inferences

To determine how many different lineages are present in the Canary Islands and infer the number of independent colonisation events, we implemented a Bayesian inference (BI) method to account for uncertainty in the underlying phylogenetic trees, in MRBAYES v3.2.2 (Ronquist et al. 2012), as implemented in the CIPRES Science Gateway v3.3 (Miller et al. 2010). We first analysed each gene fragment separately. We specified a model *a priori* allowing for the estimation of base frequencies, the proportion of invariable sites and rate-variation across sites with a gamma distribution. We used reversible-jump Markov chain Monte Carlo (MCMC) to integrate over the pool of all 203 possible reversible 4×4 nucleotide models. For the COI we specified a partitioned model based on non-saturated codon positions (1st and 2nd codon positions). All parameters were unlinked across partitions except topology and branch length, and each partition also had separate relative rate multipliers to account for variation in evolutionary rates across partitions. Other priors and settings were left as default. One hundred million MCMC generations were sampled every 1000th step and the first 25% were discarded as burn-in. We ran two independent runs each with 1 cold and 3 heated chains (T=0.04) and pooled the samples after burn-in was removed. Mixing and convergence of each run were monitored through the statistics provided in MRBAYES [values of standard deviation of partition frequencies (<0.01), potential scale reduction factors (PSRF) (1.00), effective sample sizes (ESS) (>200)] and in TRACER v1.6 (Rambaut et al. 2014). In addition, the same analysis was also performed on a concatenated dataset including 28S and non-saturated COI, specifying a partitioned model based on genes and codon positions. All other parameters were set as described above. The 18S was not included in the concatenated dataset due to its low resolution at this taxonomic level (see below).

To further explore the evolutionary intraspecific relationships between the mtDNA (COI) haplotypes within the two main Canary clades (A and B, see Results), we constructed separated phylogenetic networks using the Neighbor-Net (NNet) network method (Bryant and Moulton 2004) as implemented in SPLITSTREE v4.0 (Huson and Bryant 2006), over all three COI codon positions, based on uncorrected distances. These datasets included specimens with haplotypes not represented in previous datasets (i.e. specimens from *S. occidentalis* from La Gomera island) given that they were not successfully amplified for the 28S. Estimates of evolutionary divergence for COI of pairwise uncorrected differences (*p*-distance) between and within the Canary clades, and between the Canary clades and their respective sister taxa, were made in MEGA v6 (Tamura et al. 2013).

Divergence time estimates and parasite biogeographical history

Time calibration was performed through a Bayesian-MCMC joint estimation of phylogeny and divergence times in BEAST v2.3.0 (Bouckaert et al. 2014), using the concatenated 28S and non-

saturated COI dataset (1st and 2nd codon positions) without outgroups. Following previous model definitions implemented in MRBAYES, estimates of all three components of the site model were inferred during the MCMC analysis, using reversible jump. The method is implemented in the bModelTest package of BEAST (Bouckaert 2015). However, instead of sampling through the 203 reversible models, we restricted the set of substitution models such that we only allowed grouping within transitions and within transversions (with the exception of model 111111, where all rates are grouped), sampling only 31 of those models. We assumed that the partitions share the same evolutionary rate for each branch, and hence the same clock model. To test for clock-likeness, an initial run was set with an uncorrelated lognormal relaxed clock with substitution rate set to 1.0. The results indicated a coefficient of variation of 46.48%, which implies substantial substitution rate heterogeneity among lineages. Therefore the strict clock was rejected, and an uncorrelated lognormal relaxed clock was used for the subsequent analyses. We then tested two rates models: the Yule constant speciation rate model and no extinction (Yule 1925); and the birth–death constant speciation and extinction rates model (Nee et al. 1994; Gernhard 2008). Because a high extinction rate is a plausible scenario to account for in our system, a separate analysis was also performed assuming for the birth–death model, a relative Death Rate with high extinction rate prior density with beta distribution (Alpha = 1.0 and Beta = 4.0). We specify a diffuse “uninformative” but proper prior for the mean rate with a gamma distribution (Alpha = 0.001 and Beta = 1000) and a standard deviation rate with an exponential distribution (Mean = 0.333). Fossil calibrations are not available for these taxa. Usually in island systems, in the absence of fossil record, studies rely on relative geological dating, based on the emergence of an island as hard bounds (i.e. Cox et al. 2010). However, since we identified several lineages in the same islands, such assumption could not be followed. Instead, we assumed that the most recent common ancestor (mrca) of the Cape Verde lineage would have diverged by the time of the divergence of the ancestral Cape Verde *Tarentola* host, around 7.73 Ma (Vasconcelos et al. 2010). This divergence date was used to constrain the age of the mrca of the Cape Verde lineage (constrained to be monophyletic) as a prior with a normal distribution (standard deviation = 1.0, offset = 0). Default prior distribution settings were assumed for all other parameters. Three independent MCMC analyses were run for 100 million generations with a sampling frequency of 10 thousand, for each of the datasets. Convergence diagnostics were examined for the combined runs in TRACER v1.6 (Rambaut et al. 2014). For all the analyses, we assessed whether our prior information was not affected by interactions between priors. After verifying that all three runs converged on the posterior distributions and became stationary, we combined the sampled trees into a single file and summarized the results in LOGCOMBINER v2.3.0 (Bouckaert et al. 2014), discharging the first 25% of the samples in each tree file. Most probable trees were summarized into a maximum clade credibility tree and 95% confidence intervals of ages were calculated using TREEANNOTATOR v2.3.0

(Bouckaert et al. 2014).

Global-fit cophylogeny

We evaluated the level of coevolutionary congruence between the clade A nematodes and their respective hosts using the Procrustean Approach to Cophylogeny (PACo) (Balbuena et al. 2013) statistical tool in R v3.2.2 (R Core Team 2015). This cophylogenetic method has the advantage of not requiring resolved phylogenies and allowing for multiple host-parasite associations. This analysis was only performed for the clade A due to the restricted host use and sample sizes of the other Canary clades. We used as input the COI parasite and 12S host datasets and the respective binary matrix coding the host-parasite associations. A Procrustes superimposition plot was produced enabling a graphical visualisation of the fit of the parasite phylogeny onto the host phylogeny, as well as a goodness-of-fit statistic, whose significance was established by 100,000 randomizations of the host-parasite association data. The contribution of each individual host-parasite association to the global fit was measured by means of jackknife estimation of their respective squared residuals, together with a 95% confidence interval associated with each host-parasite link.

Descriptive parameters of parasitism

To evaluate the level of parasitism expressed at the host species level, we calculated the prevalence and mean intensity. Parasite prevalence was calculated as the ratio between the number of infected host individuals and the total number of sampled host individuals, and parasite mean intensity as the mean number of parasites per infected host. Given the differences in detectability and abundance of nematodes depending on the origin of the samples [see Jorge et al. 2013a (Appendix A)], these parameters were only calculated for the samples collected from intestines unless otherwise stated. Because only a subset of parasite specimens were sequenced, estimates of prevalence and intensity are based strictly on morphological identification of the parasites recovered, meaning that in some cases the presence of other parasite species may have been overlooked.

Results

Parasite datasets included in the phylogenetic analyses consisted of 47 sequences for 18S, 56 sequences for 28S and 57 sequences for COI, excluding outgroup sequences. Final sequence lengths for each marker were of 774bp for 18S, 1078bp for 28S and 601bp for COI. The concatenated COI and 28S datasets included 57 taxa, 29 of which representing all the main parasite haplotypes found in the Canary Islands. Xia tests indicated substantial saturation at the level of third codon positions of COI either assuming a symmetrical topology (Iss < Iss.cSym, P =

0.0727 for N=16), or an asymmetrical topology (N=8 Iss < Iss.cSym, $P = 0.2026$; while for N = 16 or 32 Iss > Iss.c, $P = 0.005$). This saturation was also evident in plots (Data B2.3, Supporting Information), with transversions outnumbering transitions for sequence pairs for uncorrected *p*-distances greater than 0.44 or JC69 distances greater than 0.70. However, no saturation was observed when accounting for each of the Canary clades separately. The datasets for the phylogenetic network reconstruction for the two main Canarian clades consisted of 30 specimens for clade A and 18 for the dataset consisting of clade B (15 specimens) plus specimens of the closest non-Canarian taxa (3 specimens). Prevalences and intensities for each of the *Spauligodon* clades from the Canary islands are reported in Table 2.1.

Table 2.1 Prevalence, average intensity (intestines) and range for each of the *Spauligodon* clades.

Clade	Island	Host	N.Host	N.Inf.Host	Prevalence	Intensity	Range
A1	Fuerteventura	*Gallotia*	9	8	0.89	73	3-287
		*Tarentola**	21	1	0.05	1	
	Tenerife	*Gallotia*	50	24	0.48	24.29	1-162
		*Tarentola**	24	1	0.04	8	
	La Palma	*Gallotia*	30	5	0.17	24.6	1-92
	La Gomera	*Gallotia*	30	13	0.43	16.31	1-114
	El Hierro	*Gallotia*	20	8	0.4	70.25	1-185
	Overall		139	58	0.42	35.59	1-287
A2	Tenerife	*Chalcides*	20	3	0.15	20.33	1-38
	La Gomera	*Chalcides*	10	6	0.6	52.667	2-109
	El Hierro	*Chalcides*	20	13	0.65	46.846	5-168
		*Gallotia**	42	1	0.024	28	
	Overall		50	22	0.44	44.82	1-168
B	Lanzarote	*Gallotia*	34	7	0.21	5.86	1-16
		*Tarentola**	30	1	0.03	5	
	Fuerteventura	*Gallotia*	27	6	0.22	6.67	3-13
	Overall		61	13	0.21	6.23	1-16
D	Gran Canaria	*Tarentola*	20	6	0.3	107.5	5-262
		Chalcides	20	1	0.05	10	
	La Gomera	*Tarentola*	11	1	0.09	3	
	Overall		138	12	0.09		3-262
C	El Hierro	*Chalcides*	20	1	0.05	214	
		*Gallotia**	42	1	0.02	36	
	Overall		20	1	0.05	214	

Number of sampled hosts (N.Host), Number of infected hosts (N.Inf.Host); *Data only retrieved from faecal samples (not included in the overall estimations)

Phylogenetic inferences

In all the BI analyses, each separate run converged to an average deviation split of

frequencies of < 0.003. The BI from each marker produced trees that varied in the degree of resolution, with the 18S being the least informative. Overall, analysis of the concatenated dataset generated a better-supported and resolved phylogeny (Fig.2.2). The *Spauligodon* nematodes in the Canary Islands are divided in four well supported clades (posterior probability > 0.99) (Fig.2.2). However, in the phylogenetic inference from the slower evolving 18S, only three clades are recovered, with clades A and B grouping together within a larger clade. The four clades do not share a recent common ancestor, representing four independent parasitic nematode clades in the Canary Islands. Clade A includes Canary nematodes ascribed to *S. occidentalis,* infecting *Gallotia* lizards and *Tarentola* geckos and *Spauligodon* sp. infecting skinks from the western islands. Clade B includes specimens ascribed to *S. atlanticus* infecting *Gallotia* lizards from the eastern islands. Clade C includes specimens infecting skinks and lizards and is exclusive from the most western

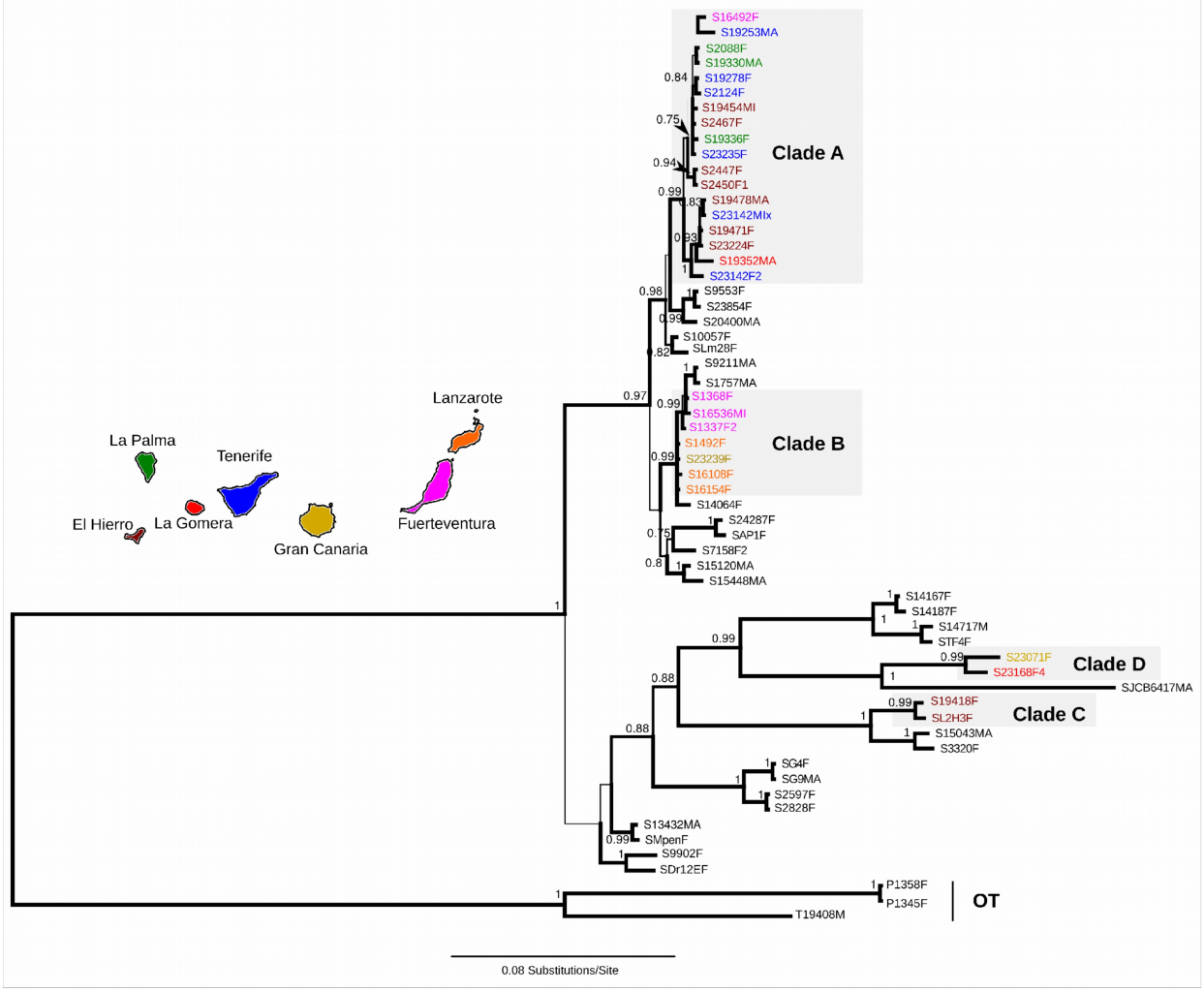

Fig.2.2- Bayesian 50% majority-rule inference tree for the concatenated 28S and COI parasite dataset. Shaded rectangles indicate Canarian parasite clades. Branch labels show posterior probabilities (values below 0.75 not shown) and branch width drawn according to the respective posterior probability support. Tip labels from each clade coloured according to the islands of origin. Outgroups (OT).

island (El Hierro). Clade D groups *Spauligodon* sp. specimens mainly present in geckos from Gran Canaria and La Gomera. Clade A is associated with parasites from Morocco, the Iberian Peninsula

and Caucasus, infecting lacertid lizards of the genera *Psammodromus*, *Timon* and *Lacerta*, respectively. Clade B groups together with other *Spauligodon* nematodes infecting *Podarcis* lizards from Morocco and northeastern Spain. Clade C clusters together with other parasites infecting skinks from Morocco and Italy, while clade D groups with *Spauligodon* infecting geckos from Mauritania and Morocco. Unfortunately, the lack of a complete phylogeny for *Spauligodon* nematodes still prevents an unambiguous phylogenetic placement for all four clades.

The NNet splits graphs for clades A and B are shown in Fig.2.3 (clade A: fit value = 97.94; clade B plus sister taxa: fit value = 97.72). The NNet of each clade highlights a predominant tree-like signal and showed some degree of reticulated structure. In clade A, the most prominent split

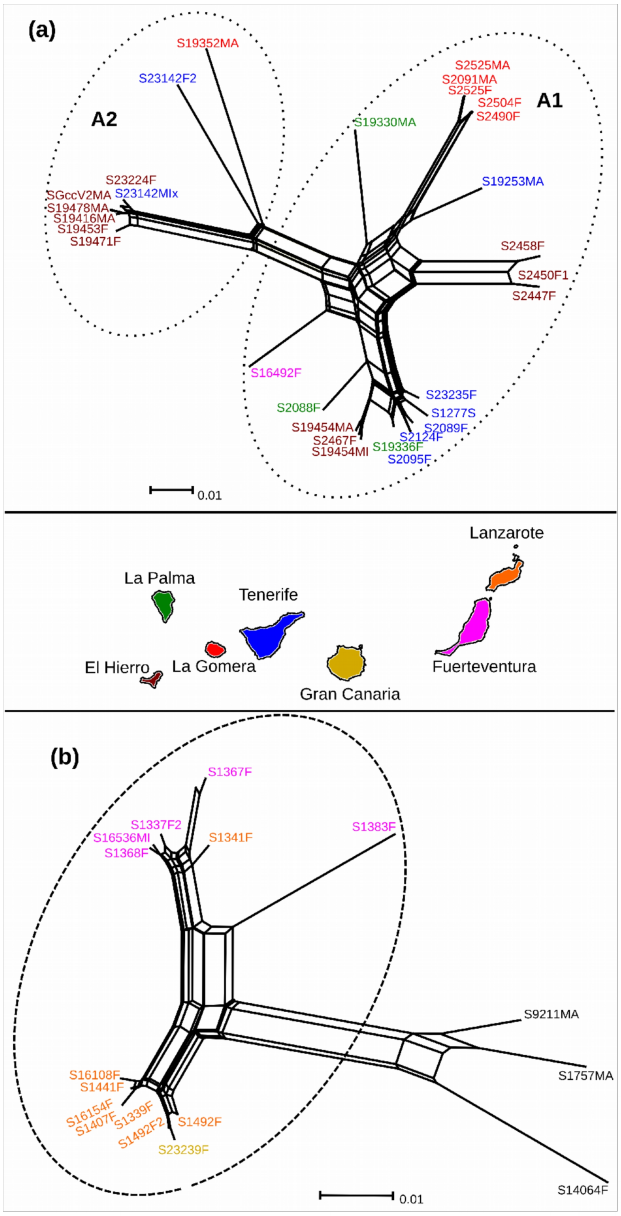

Fig.2.3- Split decomposition Neighbor-Net of the COI *Spauligodon* parasite dataset for a) Clade A with pointed line clustering the two different lineages A1 (found in lizards) and A2 (found in skinks); and b) for the Clade B (enclosed in a dashed circle) and the closest non-Canarian parasite taxa. Tip labels from each clade coloured according to the islands of origin.

separates the clade in two: A1 lineage infecting mainly lizards and A2 lineage infecting skinks (Fig.2.3a), similar to what was inferred in the concatenated BI tree. In clade B, contrary to the inferred concatenated BI tree, the lineages from *Podarcis* lizards present a clear separation from those from the Canarian lizards (Fig.2.3b). However, if we exclude the 3rd codon position from the analysis, as it is set for the concatenated dataset analysis, such differentiation disappears and the NNet splits graph collapses with no clear structure (data not shown).

Clade D was only represented by two genetically very different specimens, presenting the highest within genetic diversity (uncorrected *p*-distance of 11.6%), followed by clade A (7.7%). The estimate of evolutionary divergence between the two sub-clades of clade A was also very high (uncorrected *p*-distance of 10.5%). Between each of the four *Spauligodon* Canary clades and their respective sister taxa, we found estimates of divergence ranging from 7.5% (for Canary clade B versus Morocco and France taxa) to 18% (for Canary clade D versus one specimen from Mauritania).

Divergence estimates and parasite biogeographical history

Overall, the marginal densities for each run of the divergence time estimates analysis were nearly identical, indicating that the runs converged on the same stationary distributions. In all runs, the marginal densities for the standard deviation hyperparameter of the uncorrelated lognormal relaxed clock model were quite different from the prior, with no significant density at zero and with a coefficient of variation between 0.46 and 0.52. The phylogenetic trees had similar topology to that estimated for the combined COI and 28S in MRBAYES, with the exception of the relationships within clade B (however, posterior probability < 0.5) that were in agreement with the NNet splits graphs, demarcating the Canarian samples as monophyletic in relation to the Moroccan ones (Fig.2.4). Tree time calibrations of the combined molecular markers produced divergence time estimates slightly older under the Yule tree prior for the more recent clades, whereas for the older ones slightly younger ages were obtained, while the estimates obtained for both birth-death prior models were very similar (Table 2.2). The birth-death model had the best likelihood score (however, log likelihood difference 1.11). The time estimate to the mrca of each clade are given in Table 2.2. All the 95% highest posterior density interval (HPD) intervals for divergence time estimates get broader the further we go into the past. The marginal densities of both prior and posterior samples of the calibrated mrca of Cape Verde taxa were very similar for both the Yule model [mean: 7.58; 95% HPD intervals: 5.59-9.53; Prior mean: 7.6; 95% HPD(5.62-9.55)] and birth-death model [mean: 7.59; 95% HPD intervals: 5.56-9.53; Prior mean: 7.11; 95% HPD(5.65-9.58)].

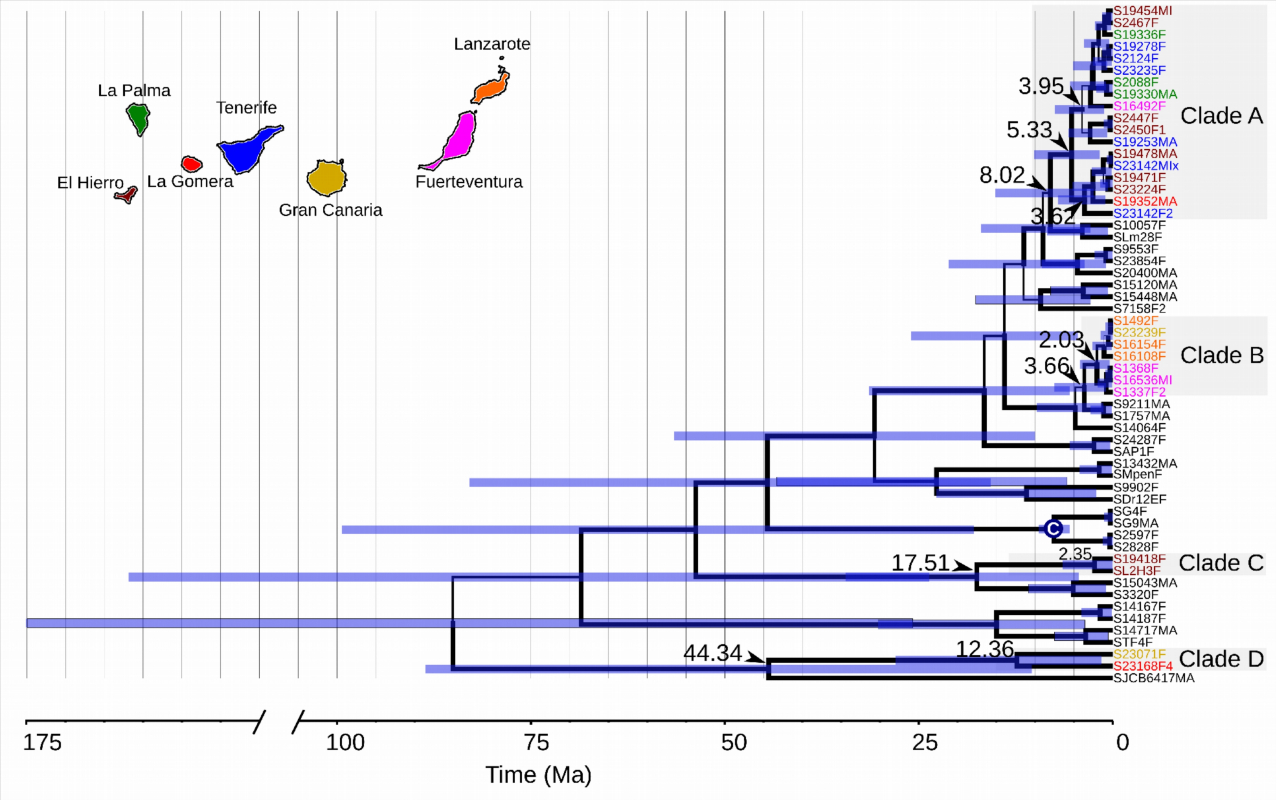

Fig.2.4- Maximum clade credibility ultrametric timescaled tree, generated under the birth-death model tree prior, for the concatenated 28S and COI parasite dataset. Shaded rectangles indicate Canarian parasite clades. Node bars represent the 95% highest posterior density intervals. Node labels show mean divergence time estimates. Branch width drawn according to the respective posterior probability support. Blue circle indicates calibration point. Tip labels from each clade coloured according to the islands of origin.

Table 2.2 Mean divergence time estimates (Mya) to the most recent common ancestor of each of the Canarian clades, with respective highest posterior density interval (HPD).

	Yule		Birth-death		Birth-death*high extinction	
Clade	Mean	HPD	Mean	HPD	Mean	HPD
A	6.5	1.8-11.55	5.33	1.67-10.15	5.55	1.53-10.58
B	2.41	0.9-8.28	2	0.4-4.25	2.15	0.42-4.52
C	2.8	0.02-7.49	2.35	0.03-6.48	2.48	0.12-4.22
D	11.15	1.69-24.6	12.36	1.49-27.97	11.99	1.51-26.66

Global-fit cophylogeny

The procrustes superimposition plot for clade A shows a high degree of fit between the parasite phylogeny and the host phylogeny, with clear segregation between the parasites infecting lizards and the ones infecting skinks (Fig.2.5). We can clearly identify the host switch event in Tenerife from a lizard to a gecko. The analysis of the cophylogeny for the clade A provides evidence for overall significant congruence between the parasite and host phylogenies (m^2 global value = 0.1893998, *P* = 0.0008). The contribution of each individual host-parasite association to the global fit was similar within the two sub-clades (Fig.2.6).

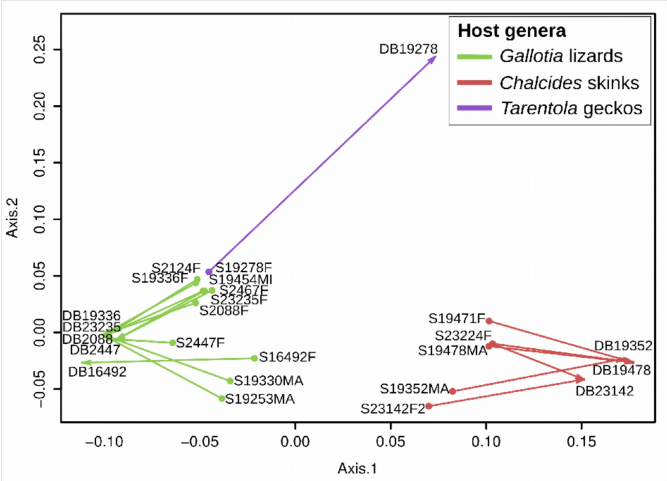

Fig.2.5- Procrustean superimposition plot for the Clade A Canarian *Spauligodon* parasites and their respective reptile hosts. Dots correspond to parasites and arrow tips to the hosts.

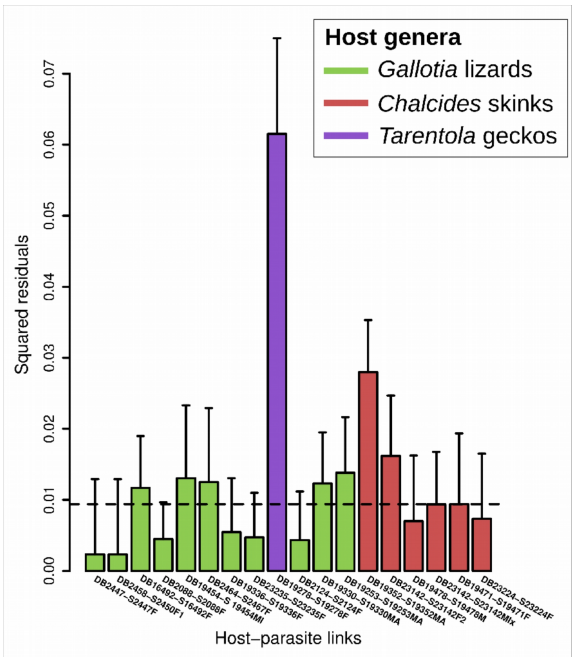

Fig.2.6- Contributions of individual host-parasite links to the Procrustean fit for Clade A. Dashed line represents the median squared residual value.

Discussion

In this study, we infer the origin of *Spauligodon* parasites in the Canary Islands and follow their evolutionary dynamics in the archipelago. Island colonisation may represent an oscillation period in the evolutionary history of *Spauligodon* parasites. In a first instance, as obligate direct life cycle parasites, colonisation of the Canarian archipelago was only made possible by “not missing the boat”, as these nematodes are completely dependent upon their host colonisation. Post colonisation, parasite diversification was determined by a combination of several evolutionary

events. In a previous study, Jorge et al. (2011) uncovered the relationships for a smaller subset of taxa, including only the parasites infecting *Gallotia* lizards, where two independent lineages were identified, leading to their separation in two non-related species [Jorge et al. 2013b (Chapter 5)]. The present analyses of a significantly expanded dataset revealed a more complex colonisation scenario for this parasitic nematodes.

How many independent colonisation events?

Our phylogenetic evidence suggests that the *Spauligodon* nematodes from the Canary Islands evolved from at least four different lineages, but maybe only representing three independent colonisation events (Fig.2.7). It is worth stressing that these parasitic nematodes rely

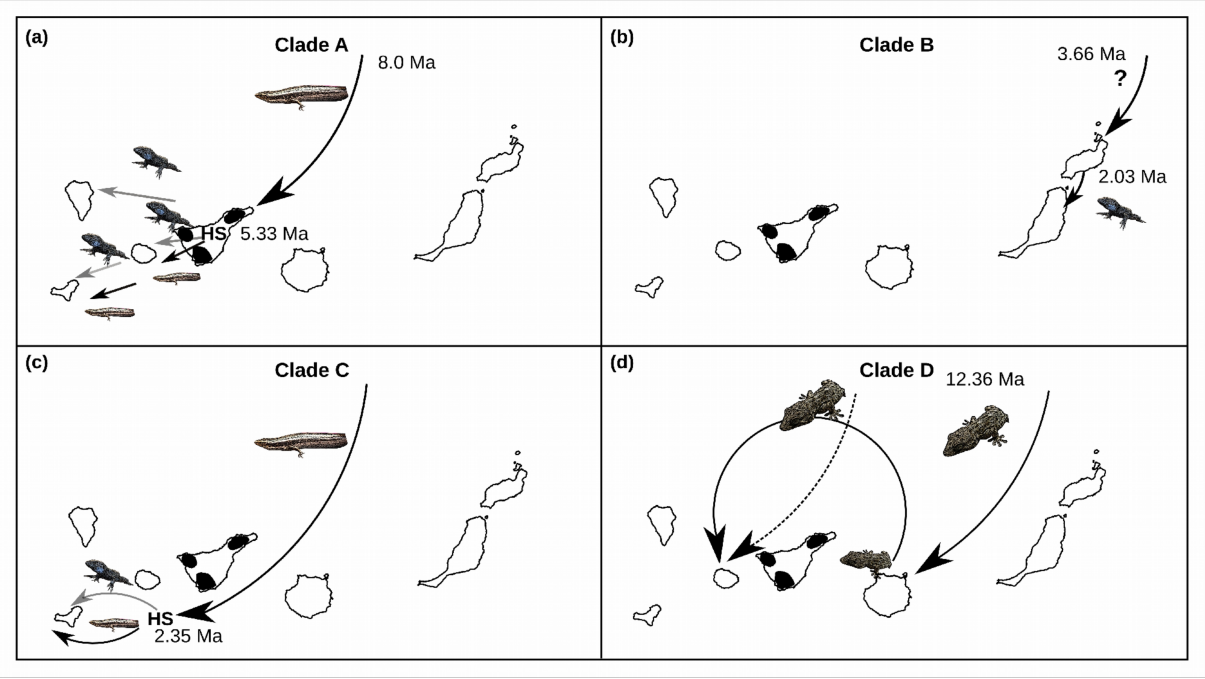

Fig.2.7- Colonisation hypotheses for the origin and diversification of each *Spauligodon* parasite clade in the Canary Islands with estimated divergence times: a) Clade A (grey lines represent new lineage after host switch (HS) event); b) Clade B; c) Clade C (Grey lines represent new lineage after host switch event); and d) Clade D (dashed line represent alternative scenarios). Areas shaded in black in Tenerife Island represent the three shield volcanoes that later gave origin to the island.

completely on their hosts to disperse (Adamson 1990), meaning that colonisations are conditioned (but not mirrored) by the host colonisation history. While it is estimated that for the reptile hosts there were at least six independent colonisation events (Carranza et al. 2002; Carranza et al. 2008; Cox et al. 2010), they could have reached the island "empty" of *Spauligodon* parasites.

When, where and "inside" who did the parasites colonise the islands?

Clade A: The mrca of clade A seems to have originated around 5.5 Ma, and soon after diverged in two lineages: one now present in the western lizards and the other most commonly

found in the western skinks (Fig.2.4). According to previous studies, divergence time estimates for the reptile hosts suggest that the lizards colonised the Canary islands 17 to 20 Ma, reaching the western-most islands around 9 to 10 Ma (Cox et al. 2010), while the ancestor of the western skinks arrived to the central and western islands around 7 Ma (Carranza et al. 2008) (Fig.2.1c and e). However, the mrca shared with other non Canarian *Spauligodon* lineages is estimated to have occurred 8.02 Ma, which excludes the lizard as a possible ancestral host. This estimation agrees with the current parasite distribution, which are absent in the older eastern islands that were not colonised by the skink taxa present in the western islands. Interestingly, we did find this parasite clade in the south of Fuerteventura island. However due to its position in the inferred phylogeny and the reported occurrence of introductions of lizards in Fuerteventura from North of Tenerife in 1980 and 1985 (Mateo et al. 2011), it seems more plausible that the presence of the parasite on this island resulted from a host-switch between recently introduced and native lizards. The most parsimonious scenario assumes that the lineage originally arrived to La Gomera or Tenerife (the only western islands already emerged during that period) with the mrca of the western skinks, and soon after host-switched to the lizard and evolved separately since (Fig.2.7a). The overall phylogeography of the two lineages of this clade shows a high degree of congruence with that of their respective hosts, suggesting a high degree of host specificity.

Clade B: The evolutionary history of this clade is very intriguing. For a start, this parasite clade only occurs in the oldest islands of the archipelago, Lanzarote and Fuerteventura, but it appears as the youngest clade. The older age of these islands certainly increases the probabilities of extinction (MacArthur and Wilson 1963; Sanmartín et al. 2008). There are three reptile hosts present on the islands, a gecko, a lizard and an endangered skink, which are estimated to have colonised these islands 3.63 to 6.30 (Rato et al. 2012), 17 to 20 Ma (Cox et al. 2010), and 5 Ma (Carranza et al. 2008), respectively. However, this parasite clade seems to have diverged approximately 3.7 Ma ago from its closest relatives which are present in Morocco (uncorrected *p*-distances = 11%) infecting *Podarcis* lizards. Later, 2.03 Ma, it diverged into two lineages, one now present in Lanzarote and the other in Fuerteventura islands, which roughly coincides with the divergence between their current host sub-species (1.52 to 4 Ma; Cox et al. 2010). Hence, the most probable scenario is that the mrca of this parasite clade did not colonise the islands with the mrca of the lizards but instead resulted from a host-switch before the two current lizard sub-species diverged (Fig.2.7b). One hypothesis would require a colonisation of *Podarcis* lizards in the Lanzarote or Fuerteventura islands, which would then have gone extinct while their parasites survived by host-switching. However, there are neither *Podarcis* lizards nor sister taxa in the Canary Islands, nor any fossil record suggesting an extinction. Therefore, this hypothesis seems too complex to be plausible. Another possibility could be that the clade B mrca colonised those islands together with

the mrca of one of the other reptiles currently present on the islands: the gecko or the skink. The prevalence in geckos is only of 3 %, with only one specimen found infected, and maybe this just indicates an occasional host-switch event. Unfortunately, due to the endangered status of the skink species in these two islands, we were not able to obtain samples of their parasite fauna. Nevertheless, we cannot completely rule out the hypothesis that the closest sister taxon for this clade remained unsampled, obscuring the origin of this clade.

Clade C: This clade was only found in the youngest island of the archipelago, El Hierro, at a very low prevalence (5% in skinks and 2% in lizards). Whether the parasite went extinct in the other islands or its low prevalence resulted in a false absence, remains unclear. The closest related taxa for this parasite clade are other *Spauligodon* nematodes infecting skinks from Morocco and Italy (estimated divergence of 13.3%, uncorrected *p*-distance), suggesting that this clade may have colonised the Canary island together with the mrca skink host 7 Ma, and later colonised El Hierro. The estimated divergence between the two lineages, the one present in skinks and the other in lizards, is older than the emergence of El Hierro island (2.35 Ma versus 1.12 Ma, respectively), meaning that similar to clade A, a host-switch has probably occurred between skink and lizard prior to their colonisation of El Hierro island (Fig.2.7c). However, this clade is only represented by two specimens, limiting our ability to safely infer its colonisation history.

Clade D: This clade was mainly found in geckos from Gran Canaria (prevalence = 30%) and La Gomera (prevalence = 9%) islands, with only one specimen found infecting a skink in Gran Canaria (prevalence = 5%). The most parsimonious scenario is that the mrca of clade D colonised the archipelago together with the mrca of geckos from central and western Canary Islands. The colonisation of this host group is fairly complex (Fig.2.1d), but it seems that the central and western islands had two separate colonisations: one 5.3 to 6.7 Ma by *T. boettgeri* ancestral to the Selvages, Gran Canaria and El Hierro; and another, 4.1 to 8 Ma by the ancestor of *T. delalandii* and *T. gomerensis* to the western islands of Tenerife, La Gomera and La Palma. In our estimated phylogeny the lineages present in Gran Canaria and La Gomera are monophyletic, but their mrca dates back to 12.36 Ma, before those islands emerged. This divergence time together with their host colonisation history, suggests that this clade may actually represent two independent colonisations (Fig.2.7d). However, the small sample size makes it difficult to uncover its colonisation history in the Canary Islands.

Which events explain the current diversity?

The complex evolutionary history of *Spauligodon* parasites in the Canary Islands was clearly underestimated in the initial study of Jorge et al. (2011) which only focused on *Gallotia* lizards as

hosts. Our current study highlights possible forces behind parasite diversification in the Canary Islands, and curiously it seems that the lizard was probably not the original ancestral host of any of the four initial *Spauligodon* lineages that colonised the archipelago. The mosaic structure of *Spauligodon* diversity in the Canary Islands (Fig.2.7) is mainly explained by a combination of ecological fitting and association by descent, with a high degree of host specificity between host and parasite lineages. Such complex evolutionary history would probably remain similar even considering that we may be underestimating the number of successful colonisation events (if such representatives were not sampled or have gone extinct); or that the time of colonisation and ancestral host hypothesised according to the divergence time estimates may be incorrect (since they heavily depend on the quality of the calibration, as highlighted in the 95% HPD). Environmental change is known to play a central role in both the persistence and diversification of host–parasite systems (Hoberg et al. 2012). New habitats create opportunities for host switching during periods of geographic expansion, while co-differentiation with hosts may occur during periods of geographic isolation or long periods of phenotypic stasis (Nieberding et al. 2008; Hoberg and Brooks 2008, 2010). Our study system provides good evidence of such dynamics over parasite evolutionary time. As obligate parasites, dispersion between and within islands is primarily limited by the dispersion abilities of the hosts. However, during the initial colonisation period, the parasite extended its host range by host switching to a different host, through means of ecological fitting. The aggregated nature of nematode population structure, together with the expected initial low host densities, may have created the conditions for interrupted gene flow between the parasite populations. The development of host specificity was later the main event that restricted host use. High host specificity is clearly seen in our system, where the different reptile hosts occur in sympatry, currently attaining high population densities and overlapping in microhabitat use. Under these conditions, one might expect that all reptiles are exposed to parasite eggs in a similar way, and host-switch events could be relatively common. However, the reptiles exhibit different ecology (diurnal and ground-dwelling in lizards, semi-fossorial in skinks and nocturnal and saxicolous in geckos, Mateo et al. 2011). Following the concept of “filters” symbolizing the mechanisms responsible for the formation of host ranges (Combes 2001), reptile behaviour and ecology may shape the encounter filter between reptile host in an asymmetrical way among the three different host groups. Host physiology may then create an additional and important filter (“compatibility filter”) explaining the observed restricted host range of each parasite species. Currently, we still observe occasional host switches (e.g. between lizards and geckos). Are these “occasional” host switches (when assuming a high degree of host specificity) indicative of the resilient plasticity of the parasites to colonise new hosts and/or expand their geographic range by ecological fitting?

Conclusion

In this study, we aimed to determine which evolutionary events have played a key role in parasitic nematode species diversification in islands system. Parasites are complex resource users, as they explore their environment at two different scales: first, their host and, second, the geographic habitat. Our study parasite taxon, the nematode *Spauligodon*, seems to have colonised the Canary Islands in at least three independent events. In this system, we identified the dynamics between island colonisation, as an example of an oscillation stage in the parasites' evolutionary history, and diversification, mainly explained by ecological fitting followed by a period of host specialisation. The conservativeness in host use restricts but does not prevent the colonisation of new hosts. Such rare events of successful host-switching have enabled the parasites to successfully colonise new habitats and hosts, avoiding extinction in episodes of environmental change such as island colonisations, and promoting an initial niche enlargement and ultimately niche shifts. In this parasite system, the diversification of host use represents the main source of parasite diversification, providing raw material for ensuing speciation processes. Rather than defining such incongruence in host use as the Parasite Paradox, we prefer to use it as an example of why parasites have such a successful lifestyle.

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Supporting information

Additional Supporting Information can be found in the Appendix B.

Data B2.1. Nematode specimens used in the phylogenetic analyses, including their respective host species, locality and GenBank accession numbers.

Data B2.2. Reptile specimens used in the cophylogenetic analysis, including their locality and GenBank accession numbers.

Data B2.3. Saturation plots for the COI third codon positions.

CHAPTER 3

New host, new rate? A perspective of the rate of molecular evolution in *Spauligodon* (Nematoda) parasites.

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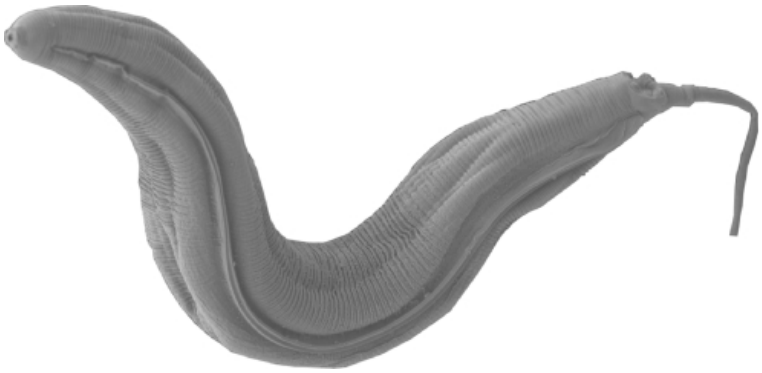

Spauligodon atlanticus Type Mor, adult male

Abstract

Unravelling which factors correlate with evolutionary rates will enable a better understanding of the evolution of organisms. Parasites present high rates of molecular evolution compared to their hosts and non-parasitic relatives, which are expected to result from their life cycle peculiarities. In this study we evaluated if the coevolutionary events that have shaped the parasites evolutionary history influence the rate of molecular evolution at the whole genome-level. We analysed mitochondrial and nuclear DNA sequences from parasitic nematodes belonging to the genus *Spauligodon*, and estimated the relative rates of evolution with a Bayesian method. Host-parasite phylogenies were then analysed with a global fit approach. Parasite associations were split into two groups; lineages showing topology congruence with their ancestral hosts, as an approximation to codivergence, and those showing topology incongruence resulting from switches to new, unrelated hosts. We tested if the estimated rates of evolution for each parasite lineage were correlated with the level of congruence between each host-parasite link and the time since the origin of each association. Our results did not detect significant differences in rates between congruent and incongruent parasite lineages, nor did we find that the degree of incongruence between topologies or time influence the parasite evolutionary rate. It remains unclear whether these results arise from a real independence between rates and historical events in host-parasite coevolutionary associations, or such dependence only applies to a selected range of genes directly involved in host-parasite interaction, with no influence of demographic events. Nonetheless, this study offers new insights into factors that may influence the rates of molecular evolution in parasites.

Introduction

One of the major challenges in evolutionary biology is understanding the underlying factors influencing the variation in rates of molecular evolution across the tree of life (Lanfear et al. 2010). How the rate at which mutations arise per genome relates to the tempo of evolutionary change within a population, ultimately reflecting in the rates of nucleotide substitution (Duffy et al. 2008; Barrick and Lenski 2013). Molecular rate heterogeneity has been reported within and among species clades, uncovering extreme deviations from a clock-like assumption. Several studies have focused on finding which biological traits correlate with rates of molecular evolution, such as DNA repair (Ying et al. 2010), population size (Woolfit 2009), body size (Bromham 2002) and generation time (Smith et al. 2008; Thomas et al. 2010). Some general trends have been observed, namely, smaller organisms, with short generation times and high reproductive output, generally attain faster relative rates of molecular evolution; whereas bigger animals with longer generation time have slower rates.

Due to their particular lifestyle, parasites are expected to have rapid evolution. Parasites seem to evolve faster than their host (Haraguchi and Sasaki 1996) and faster than their non-parasitic relatives (Bromham et al. 2013). This increase in parasite evolutionary rates may be explained by the shorter generation times, smaller population sizes and also frequent demographic bottlenecks (Ebert 2008; Bromham et al. 2013). Parasites may be "mutating for their life" during host-parasite arms race, in which selective advantage for higher mutation rate will still exceed the costs of deleterious mutations (Haraguchi and Sasaki 1996; but see Loverdo and Lloyd-Smith 2013). This hypothesis will assume a gene-for-gene or allele-matching models of coevolutionary dynamics of host-parasite interactions, with an antagonistic interaction between host and parasite genotype (Haraguchi and Sasaki 1996; Tellier et al. 2014). In such case, an increase in rates will only be observed in those genes involved in the antagonistic interaction. However, not only genes directly involved in successful host exploitation can be selected to raise the substitution rate, a whole genome-level increase in rates may occur (Barrick and Lenski 2013).

Rates of molecular evolution may vary between different parasite species, influenced by relatedness (phylogenetic inertia) as in non-parasitic organisms. Generally, parasites are expected to be locally adapted (Gandon and Micalakis 2002) and host-specific (Dick and Patterson 2007). For example, in maternally transmitted parasites (i.e. parasites transmitted from mother to offspring) selection seems to act against higher mutation rates in the parasites to maintain similar parasite infectivity, consequently weakening selection for increased mutation rates (Greenspoon et al. 2013). In a similar way, under a "stable" coevolving host-parasite interaction parasites may present relatively lower rates of molecular evolution while maintaining the local adaptability. However, host-parasite interactions are dynamic and in reality host-parasite codivergence seems to be the exception rather than the rule, with host switches being common (de Vienne et al. 2013). While host switches between closely related host species is to some extent expected (Longdon et al. 2014), host shifts to relatively unrelated hosts also occurs (Ricklefs et al. 2004; Brooks et al. 2006; Duval et al. 2007). What are the evolutionary consequences for parasites of host codivergence versus host shift? Will the changes in demography and/or new selective pressure to out-evolve and adapt to a new host consequently alter the rate of molecular evolution? A new host-parasite association will be initially characterised by a population size bottleneck. In endosymbiotic microorganisms, reduction in effective population size has been associated with increased substitution rates (Woolfit and Bromham 2003). Following the population bottleneck, after a successful host-switch, the parasite may increase their population size. Such changes in population size are known to affect the evolutionary rates at a whole genome-level (Charlesworth 2009). Additionally, higher mutation rates should be favoured in host switches so the parasite can evolve to successfully exploit the new host, as oppose to a codivergence scenario where the parasite is expected to be already locally adapted. There are a few examples of such variation in

evolutionary rates between antagonistic interactions with a newly acquired partner and those involving a historical partner (i.e. adapted) in phages (Paterson et al. 2010) and in animal viruses (Einer-Jensen et al. 2004). However, such pressures may or may not lead to a whole genome-level change in rates. The time since the host switch event also needs to be considered when evaluating its effects on parasites evolution, since following a host switch event parasites can still specialise and adapt to the new host (Little et al. 2006).

The rate of molecular evolution can be estimated by determining the number of substitutions that have occurred in different lineages (branch lengths) using a model of evolution (Lanfear et al. 2010). Measuring the substitution rates at sites that are not constrained by selection, directly provides information on the mutation pattern (Duret and Mouchiroud 2000). In protein-coding sequences, a change in the mutation rate will affect the occurrence of synonymous and nonsynonymous mutations equally. Due to differential selective pressures on nonsynonymous substitutions, synonymous substitutions are expected to reflect the underlying mutation rate. (Thomas et al. 2010). However, accurate rate estimates may then be conditioned given problems of saturation at synonymous sites (Lanfear et al. 2010; Thomas et al. 2010).

In this study, we investigated the effect of the parasites' evolutionary history on the rate of molecular evolution, assuming that different evolutionary events would have an effect at the whole genome-level. Our model organism is a parasitic nematode, genus *Spauligodon* that infects the intestines of reptiles. This parasite is a haplodiploid (males deriving from non-fertilised eggs whereas females derive from fertilised eggs) and has a direct life cycle (Adamson 1990). Transmission occurs with nematode eggs being deposited together with host faeces, presenting overall low dispersal ability. The aggregated population structure of this parasite may favour transmission among related hosts where lower mutation rates may be expected. However, host-switches to relatively unrelated hosts have occurred throughout their evolutionary history (Chapter 2) and consequently, variation in molecular rates may have occurred due to changes in population sizes, and/or pressures to successfully exploit the new hosts.

We hypothesised that parasite lineages evolving in congruence with host phylogeny will present an overall slower evolutionary rate, in contrast to those resulting from switches to new, unrelated hosts which will have relatively higher rates. We assume that such historical events will have a whole genome-level influence, so changes in rates would be also detected in neutral genes. The rate variation and relative divergence time were estimated for *Spauligodon* parasitic lineages by means of Bayesian Markov chain Monte Carlo coalescent framework. To characterise different events in the evolutionary history of the parasite, a global fit method was used to assess the degree of congruence between parasites and host topologies. We then assessed if the level of host-parasite phylogenetic congruence and the relative divergence time of each lineage (as an approximation to age of the host-parasite association) influenced the estimated rates of molecular

evolution.

Material and Methods

Molecular data

Parasite specimens were collected from faecal pellets and intestines from several reptile hosts distributed along the Mediterranean region and Macaronesian Islands (Table 3.1). Samples were collected and processed as described in Jorge et al. [2014 (Chapter 4)]. Extraction of genomic DNA was performed on individual nematodes using the PureLink® Genomic DNA Kit (Invitrogen, Invitrogen New Zealand Ltd, Auckland, New Zealand) according to the manufacturer's protocol. We included four DNA fragments in our analyses: three nuclear fragments, the 18S ribosomal RNA (18S), the 28S ribosomal RNA (28S) and the internal transcribed spacer 1 (ITS1) and one mitochondrial gene, the cytochrome oxidase subunit I (COI). The 18S was amplified using the primers Nem 18S F and Nem 18S R from Floyd et al. (2005). For the amplification of the 28S fragment, primers 28S rD1.2a and 28S B described by Whiting (2002) were used. ITS1 was amplified with rDNA2 (Vrain et al. 1992) and rDNA1.58s (Cherry et al. 1997) primers. The COI fragment was amplified using the nematode cocktail primers C_NemF1_t1 and C_NemR1_t1 from Prosser et al. (2013). Polymerase chain reactions (PCR) were performed in a total volume of 20 µL, comprising 4 µL of MyTaq TM Red reaction buffer (Bioline, Bioline (Aust) Pty Ltd, Alexandria, NS W, Australia), primers at 0.5 mM each, 0.1 µL of MyTaq TM Red DNA Polymerase (Bioline) and 2–3 µL of extracted nematode DNA template. For all the sets of primers, PCR consisted of 35 iterations of the following cycle: 40 s at 95 °C, 40 s at 45–58 °C (depending on the primers used) and 1 min at 72 °C, beginning with an additional denaturation step of 3 min at 95 °C and ending with a final extension at 72 °C for 10 min. Amplified 18S, 28S and ITS1 fragments were sequenced for both strands with the same primers used in the amplification process, whereas for COI, following Prosser et al. (2013), the primers M13F and M13R (Messing 1993) were used. PCR product purification and sequencing was performed by a commercial facility (Macrogen Corporation, http://www.macrogen.com/).

Parasite sequence chromatograms were edited and trimmed in GENEIOUS v8.1.4 (http://www.geneious.com, Kearse et al. 2012). We then gathered additional *Spauligodon* DNA sequence data published in previous studies (Table 3.1). However, for all the samples from Jorge et al. [2011 and 2014 (Chapter 4)] we additionally amplified the ITS1 nuclear fragment. For 18S analysis we assigned three outgroups: *Parapharyngodon* spp., *Thelandros* sp. and *Skrjabinodon* sp. (Table 3.1). The nematode *Skrjabinodon* sp. was the only outgroup used in the other datasets. Datasets of each nuclear DNA fragment were aligned using MAFFT (Katoh et al. 2002), in CIPRES Science Gateway v3.3 (Miller et al. 2010), using a preconfigured MAFFT strategy that considers

Table 3.1 Nematode specimens used in the phylogenetic analyses, including their respective host species, locality and GenBank accession numbers.

Code	Species	GenBank				Reference
		18S	ITS1	28S	COI	
KF029009	*Spauligodon anolis*	KF029009	-	-	-	Falk and Perkins 2013
KF029048	*Spauligodon anolis*	KF029048	-	-	-	Falk and Perkins 2013
S1492F	*Spauligodon atlanticus*	-	x*	KF029048	JF829279	Jorge et al. 2011
S14717MA	*Spauligodon auziensis*	x	x*	x	x	Chapter 2
SAP1F	*Spauligodon cabrerae*	-	x*	x	x	Chapter 2
SM2MI	*Spauligodon cabrerae*	x	-	-	-	This study
S13432MA	*Spauligodon carbonelli*	KJ778082	-	-	-	Jorge et al. 2014
S13437	*Spauligodon carbonelli*	-	x	x	x	This study
SMPendF	*Spauligodon carbonelli*	KJ778080	x*	KJ778090	x*	Jorge et al. 2014
SLm28F	*Spauligodon lacertae*	JF829237	x*	JF829255	JF829287	Jorge et al. 2011
S10057F	*Spauligodon lacertae*	JF829238	x*	JF829252	JF829286	Jorge et al. 2011
S2597F	*Spauligodon nicolauensis*	JF829226	x*	JF829243	JF829265	Jorge et al. 2011
S19454MA	*Spauligodon occidentalis*	KJ778077	x*	KJ778098	KJ778107	Jorge et al. 2014
S19330MA	*Spauligodon occidentalis*	x	x*	x	x	Chapter 2
S19253MA	*Spauligodon occidentalis*	x	x*	x	x	Chapter 2
S7456MA2	*Spauligodon paratectipenis*	x	x*	x	x	This study
S9902F	*Spauligodon saxicolae*	JF829227	x*	JF829246	JF829266	Jorge et al. 2011
FA502MA	*Spauligodon saxicolae*	x	x*	x	x	This study
SDB3F	*Spauligodon saxicolae*	x	x*	x	x	This study
SDr23	*Spauligodon saxicolae*	-	x*	x	x	This study
SDr12EF	*Spauligodon saxicolae*	x	-	-	-	Chapter 2
S11212F	*Spauligodon* sp.	x	x*	x	x	This study
S9582F	*Spauligodon* sp.	x	x*	x	x	This study
S22866F	*Spauligodon* sp.	x	x*	x	x	This study
SFA474F	*Spauligodon* sp.	x	x*	x	x	This study
S15120MA	*Spauligodon* sp.	KJ778079	x*	KJ778100	KJ778109	Jorge et al. 2014
S14561F	*Spauligodon* sp.	x	x*	x	x	Chapter 2
S24080MA	*Spauligodon* sp.	x	x*	x	x	This study
S23755F	*Spauligodon* sp.	x	x*	x	x	This study
SPsD2F	*Spauligodon* sp.	x	x*	x	x	This study
S14988F	*Spauligodon* sp.	x	x*	x	x	This study
S2637F	*Spauligodon* sp.	x	x*	x	x	This study
S14897F	*Spauligodon* sp.	x	x*	x	x	This study
S5011F	*Spauligodon* sp.	x	x*	x	x	This study
S20448F	*Spauligodon* sp.	x	x*	x	x	This study
S20571F	*Spauligodon* sp.	x	x*	x	x	Chapter 2
S16078F	*Spauligodon* sp.	x	x*	x	x	This study
S12489F	*Spauligodon* sp.	x	x*	x	x	This study
S9168F	*Spauligodon* sp.	x	x*	x	x	This study
S7930MA	*Spauligodon* sp.	x	x*	x	x	This study
S15742F	*Spauligodon* sp.	x	x*	x	x	This study
S7158F2	*Spauligodon* sp.	x	x*	x	x	Chapter 2
S22475F	*Spauligodon* sp.	x	x*	x	x	This study

x, unsubmitted sequence data to GenBank; * Reference: This study.

Table 3.1 (cont.)

Code	Species	GenBank				Reference
		18S	ITS1	28S	COI	
SJCB6417MA	*Spauligodon* sp.	x	x*	x	x	Chapter 2
SJCB6956F	*Spauligodon* sp.	x	x*	x	x	This study
S14167F	*Spauligodon* sp.	x	x*	x	x	Chapter 2
STF4	*Spauligodon* sp.	x	x*	x	x	Chapter 2
S21269F	*Spauligodon* sp.	-	x*	x	x	This study
S23071F	*Spauligodon* sp.	x	x*	x	x	Chapter 2
SG9MA	*Spauligodon* sp.	KJ778086	x*	KJ778095	KJ778104	Jorge et al. 2014
S15043MA	*Spauligodon* sp.	x	x*	x	x	Chapter 2
S19478MA	*Spauligodon* sp.	x	x*	x	x	Chapter 2
S11323F2	*Spauligodon* sp.	x	x*	x	x	This study
SP0775	*Spauligodon* sp.	x	x*	x	x	This study
SRP1008	*Spauligodon* sp.	x	x*	x	x	Mocket et al.
KF028940	*Parapharyngodon cubensis*	KF028940	-	-	-	Falk and Perkins 2013
KF029066	*Parapharyngodon cubensis*	KF029066	-	-	-	Falk and Perkins 2013
RP999	*Skrjabinodon* sp.	x	x*	x	x	Mocket et al. Unpublished
T19408M	*Thelandros tinerfensis*	KJ778073	-	-	-	Jorge et al. 2014
P1328F	*Parapharyngodon echinatus*	JF829223	-	-	-	Jorge et al. 2011
P1345F	*Parapharyngodon echinatus*	JF829224	-	-	-	Jorge et al. 2011

x, unsubmitted sequence data to GenBank; * Reference: This study.

RNA structure for the 18S and 28S and that favours accuracy for ITS1, employing the Q-INS-i algorithms, all other parameters were left as default. The most critical stage in the analysis of substitution rates is sequence alignment (Lanfear et al. 2010). To discard poorly aligned positions characteristic of ITS1 and 28S alignments, which could influence the rates estimates, MAFFT trimmed alignments were uploaded to Gblocks server (Castresana 2000, Talavera and Castresana 2007) and a less stringent analysis was carried out for each of those datasets. COI sequences were aligned in GENEIOUS v8.1.4 (http://www.geneious.com, Kearse et al. 2012) with Clustal W (Thompson et al. 1994). The COI alignment was visually inspected and translated to amino acids, specifying invmtDNA gene code, in GENEIOUS v8.1.4 (http://www.geneious.com, Kearse et al. 2012) to determine the correct reading frame. Saturation was evaluated by implementing the Xia test (Xia et al. 2003, Xia and Lemey 2009) in DAMBE v5.3.48 (Xia 2013). Complete datasets for all four markers were obtained for 50 specimens. Final sequences alignment lengths were 838bp for 18S, 920bp for ITS1, 1109bp for 28S, and 575bp for COI. Due to the shorter COI available sequence of the outgroup (403bp) relative to the ingroup sequences, difference in the final alignment (141bp at 5' and 31bp at 3') were coded as “?”. For the ITS1 and 28S, the complete alignments after the removal of poorly aligned sites were of 276bp and 915bp, respectively.

Host 12S ribosomal RNA (12S) sequences corresponding to each host-parasite link of the parasite dataset were downloaded from GenBank, excluding two of the parasites lineages for

Table 3.2 Reptiles host sequences used in the cophylogenetic analysis including their respective parasite link and GenBank accession numbers.

Parasite	Host	Host Family	Host species	Reference
SP0775	KC438505	Gekkonidae	*Phelsuma lineata*	Gehring et al. Unpublished
S23755	AY633412	Lacertidae	*Acanthodactylus erythrurus*	Harris et al. 2004 Unpublished
S7930	GQ142080	Lacertidae	*Algyroides marchi*	Pavlicev and Mayer 2009
S9582	AF440599	Lacertidae	*Archeolacerta bedriagae*	Mayer and Arribas 200
S2637	JX462070	Lacertidae	*Atlantolacerta andreanskyi*	Barata et al. 2012
S5011	JX462112	Lacertidae	*Atlantolacerta andreanskyi*	Barata et al. 2012
SFA502	AF080284	Lacertidae	*Darevskia chlorogaster*	harris et al. 1998
SDr23	DB5350	Lacertidae	*Darevskia rudis*	Freitas et al. Unpublished
S9902	DB10308	Lacertidae	*Darevskia unisexualis*	Freitas et al. Unpublished
S19253	DB19253	Lacertidae	*Galloti galloti galloti*	Chapter 2
S1492	AY151915	Lacertidae	*Gallotia atlantica*	Carranza et al. 2004
S19454	DB19454	Lacertidae	*Gallotia caesaris*	Chapter 2
S19330	DB19330	Lacertidae	*Gallotia galloti*	Chapter 2
S12489	AY256653	Lacertidae	*Iberolacerta horvathi*	Arribas et al. 2006
S20448	AF440589	Lacertidae	*Iberolacerta monticola*	Mayer and Arribas 2003
SFA474	GQ142088	Lacertidae	*Iranolacerta brandtti*	Pavlicev and Mayer 2009
SLm28	KC896865	Lacertidae	*Lacerta media*	Ahmadzadeh et al. 2013
S10057	DQ097094	Lacertidae	*Lacerta strigata*	Godinho et al. 2005
S11212	HQ898222	Lacertidae	*Podarcis hispanica*	Kaliontzopoulou et al. 2011
S20571	HQ898102	Lacertidae	*Podarcis hispanica* PH1A	Kaliontzopoulou et al. 2011
S13437	HQ898125	Lacertidae	*Podarcis hispanica* PH2	Kaliontzopoulou et al. 2011
SMPen	HQ898088	Lacertidae	*Podarcis hispanica* T1A	Kaliontzopoulou et al. 2011
SAP1	EF694767	Lacertidae	*Podarcis lilfordi*	Brown et al. 2008
S7158	EF694770	Lacertidae	*Podarcis sicula*	Brown et al. 2008
S16078	AF080279	Lacertidae	*Podarcis taurica*	Harris et al. 1998
S15120	DQ017658	Lacertidae	*Podarcis tiliguerta*	Mayer and Podnar 2005 Unpublished
S14561	HQ898230	Lacertidae	*Podarcis vaucheri*	Kaliontzopoulou et al. 2011
SPsD2	AF206588	Lacertidae	*Psammodromus algirus*	Fu 2000
S9168	GQ142074	Lacertidae	*Scelarcis perspicillata*	Pavlicev and Mayer 2009
S14988	AY277602	Lacertidae	*Scelarcis perspicillata*	Oliverio et al. 2008
S14897	DB11014	Lacertidae	*Scelarcis perspicillata*	Perera et al. Unpublished
S7456	HQ675926	Phyllodactylidae	*Hemidactylus turcicus*	Rato et al. 2011
S2597	AF186175	Phyllodactylidae	*Tarentola bocagei*	Carranza et al. 2000
S23071	F186125	Phyllodactylidae	*Tarentola boettgeri boettgeri*	Perera and Harris 2008
S14167	JQ300564	Phyllodactylidae	*Tarentola desertii*	Rato et al. 2012
SJCB6417	AF363572	Phyllodactylidae	*Tarentola ephippiata*	Carranza et al. 2002
SG9	AF186147	Phyllodactylidae	*Tarentola gigas*	Carranza et al. 2000
S14717	HM014490	Phyllodactylidae	*Tarentola mauritanica*	Rato et al. 2012
S21269	HM014515	Phyllodactylidae	*Tarentola mauritanica*	Rato et al. 2012
SJCB6956	JQ300713	Phyllodactylidae	*Tarentola mauritanica*	Rato et al. 2012
STF4	JQ300555	Phyllodactylidae	*Tarentola mauritanica*	Rato et al. 2012
S22475	AF186159	Phyllodactylidae	*Tarentola substituta*	Carranza et al. 2000
S19478	DB19478	Scincidae	*Chalcides coeruleopunctatus*	Chapter 2
SRP1008	EU567970	Scincidae	*Oligosoma aenuem*	Chapple et al. 2009
S11323	JQ686293	Sphaerodactylidae	*Quedenfeldtia trachyblepharus*	Barata et al. 2012

which only the genus of the host was not known (2 sequences) (Table 3.2). Alignment was

performed as described above for the nuclear parasite markers. Additional three host sequences were later removed from the final host dataset due to lower similarity with other sequences (<30%).

Phylogenetic inference

Bayesian inference (BI) analyses were carried out in MRBAYES v3.2.2 (Ronquist et al. 2012), as implemented in the CIPRES Science Gateway v3.3 (Miller et al. 2010) for each alignment. We specified a model a priori allowing for the estimation of base frequencies, the proportion of invariable sites and rate-variation across sites with a gamma distribution. However, we did not specify the site model, but instead used the reversible-jump Markov chain Monte Carlo (MCMC) to integrate over the pool of all 203 possible reversible 4×4 nucleotide models. For the COI we specified a partitioned model based on non-saturated codon positions (1st and 2nd codon positions). All parameters were unlinked across partitions except topology and branch length, and each partition also had separate relative rate multipliers to account for variation in evolutionary rates across partitions. Other priors and settings were left as default. One hundred million MCMC generations were sampled every 1000th step and the first 25% were discarded as burn-in. We performed two independent runs each with 1 cold and 3 heated chains (between T=0.04 and T=0.03) and pooled the samples after burn-in was removed. In addition, the same analysis was also performed on the concatenated dataset including ITS1, 28S and non-saturated COI, specifying a partitioned model based on genes and codon positions. All other parameters were set as described above. The 18S was not included in the concatenated dataset to be consistent with the dataset used in the rates estimates (see below). Mixing and convergence of each run were monitored through the statistics provided in MRBAYES [values of standard deviation of partition frequencies (<0.01), potential scale reduction factors (PSRF) (1.00), effective sample sizes (ESS) (>200)] and in TRACER v1.6 (Rambaut et al. 2014).

Relative rate and time estimates

The number of substitutions in different lineages (branch lengths) was simultaneously estimated with the topology of the phylogenetic tree using a Bayesian MCMC method in BEAST v2.3.0 (Bouckaert et al. 2014). We used the concatenated ITS, 28S and non-saturated COI dataset, partitioned by gene and by codon (excluding 3rd COI codon position). The less informative 18S (88.7% identical sites) was not included in this dataset, because sequences with a small number of substitutions are associated with higher estimate errors, since a small difference will represent a large proportional difference in the estimated substitution rate (Lanfear et al. 2010). Two different clocks were assumed: one for the nuclear and the other for the mitochondrial dataset. Preliminary analyses were indicative of substantial substitution rate heterogeneity among lineages (coefficient of variation of > 40%); therefore the strict clock was rejected. An uncorrelated

Lognormal relaxed clock was implemented to avoid any a priori assumption about the way substitutions rates can change as suggested in Lanfear et al. (2010). An absolute time scale was not required in our study since we aimed to compare rates not to infer absolute values; hence, the root was arbitrarily set to a uniform distribution between 85 and 95. The site model was inferred during the MCMC, estimating all three components of the site model using reversible jump, but grouping within transitions and within transversions (with the exception of model 111111, where all rates are grouped). The method was implemented in the bModelTest package of BEAST (Bouckaert et al. 2015). The birth–death constant speciation and extinction rates model (Nee et al. 1994; Gernhard 2008) was set as tree prior. We specified a diffuse "uninformative" but proper priors characterising the rate of evolutionary change with a gamma distribution (Alpha = 0.001 and Beta = 1000) on the mean and with an exponential distribution (Mean = 0.333) on the standard deviation. Default prior distribution settings were assumed for all other parameters. Three independent MCMC analyses were run for 100 million generations with a sampling frequency of 10 thousand. Convergence diagnostics were examined for the combined runs in TRACER v.1.6 (Rambaut et al. 2014). After verifying that all three runs converged on the posterior distributions and became stationary, we combined the sampled trees into a single file and summarized the results in LOGCOMBINER v2.3.0 (Bouckaert et al. 2014), discharging the first 25% of the samples in each tree file. Most probable trees were summarized into a maximum clade credibility tree using TREEANNOTATOR v2.3.0 (Bouckaert et al. 2014). Rate and relative divergence time information generated for each branch from the maximum clade credibility tree were visualised in FIGTREE v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/).

Host-parasite coevolutionary history

A global fit method was implemented aiming at determining the level of coevolutionary congruence between host and parasite phylogenies for each individual link. We applied a Procrustean Approach to Cophylogeny (PACO) (Balbuena et al. 2013) statistical tool in R v3.2.2 (R Core Team 2015), which does not require fully resolved phylogenies. The script was executed with the parasite 28S dataset, the host 12S dataset and the respective binary matrix coding the host-parasite associations. A Procrustes superimposition plot was produced enabling a graphical visualisation of the fit of the parasite phylogeny onto the host phylogeny. However, these distances in the two-dimensional plot underestimate the actual residuals in a full-dimension (Balbuena et al. 2013). A goodness-of-fit statistic was also calculated, whose significance was established by 100,000 randomizations of the host-parasite association data. The contribution of each individual host-parasite association to the global fit was measured by means of jackknife estimation of their respective squared residuals, together with a 95% confidence interval associated for each host-parasite. In our study we focused on the individual links that do or do not contribute to the overall

congruence between the phylogenies. Since we did not identify duplication or failure to speciate with their host events in the study system, for simplicity, incongruence will be assumed to be the result of host-switching events. According to the global fit results we classified each host-parasite association as congruent (as an approximation to codivergence) or incongruent lineages (as consequence of host-switching). Parasite lineages belonging to a host-parasite link with the mean squared jackknife residuals below the estimated median squared residual value were classified as congruent parasite lineages. Host-parasite link with the mean squared jackknife residual above the estimated median squared residual value were classified as incongruent lineages. Additionally, for a more conservative approach, we only considered as congruent lineages parasites from links which the upper 95% confidence interval was below the estimated median squared residual value.

Rate comparisons

A Mann-Whitney-Wilcoxon Test (function wilcoxon.test of the *R* package) was performed to determine if there was significant variation in the estimated mean substitution rates between congruent and incongruent parasite lineages. We further tested for correlation between estimated substitution rate means and the mean value and upper 95% confidence interval of squared jackknife residuals of each respective parasite lineage using a nonparametric Spearman correlation (function rcorr, R package *Hmisc*, Harrell 2013). To determine the influence of time since the establishment of host-parasite association, the same analysis was also used between the relative mean divergence times (as an approximation of time since the establishment of host-parasite association) for each host-parasite link and the mean rate. To test whether the mean rate could be determined by the degree of congruence and time of divergence of each lineage, we performed a generalised linear model with a Lognormal Regression function using a gaussian distribution with mean rates as response variable and the squared jackknife residuals, relative mean divergence times and their interaction as predictors. Prior to the regression analysis, the response variable was Box-Cox transformed to have a Gaussian-like distribution (function box.cox.powers, R package *car*; Fox and Weisberg 2011). For all analyses, we only considered rates and divergence times from terminal branches with posterior probability above 0.75 (n = 42). All analyses were implemented using the package R v3.2.2 (R Core Team 2015).

Results

Molecular datasets

We assembled a complete dataset for 50 *Spauligodon* parasites, infecting 41 different host species representing 18 genera, from 49 different localities. Xia tests indicated substantial saturation at the level of third codon positions of the COI fragment when assuming a symmetrical

topology (lss < lss.cSym, but $P > 0.05$ for N = 16 and 32), or an asymmetrical topology (lss > lss.cSym, $P < 0.001$ for N = 16 and 32). For the Bayesian inference (BI) analyses, each separate run converged to an average deviation split of frequencies inferior to 0.002. All specimens included in our analyses clearly group within the *Spauligodon* clade (Fig.3.1). The BI from each marker produced trees that varied in the degree of resolution, with the 18S being the least informative. In all inferred phylogenies several internal branches presented low support (i.e. posterior probability <0.75), indicative of underlying uncertainty in tree topology and/or branch length. The variation in branch lengths estimated with BI implied some variation in substitution rates. However they are still a product of rate and time. In the *Spauligodon* inferred phylogenies several closely related lineages do not infect closely related hosts, which suggests that host-switch events have occurred along the parasite evolutionary history (Fig.3.2).

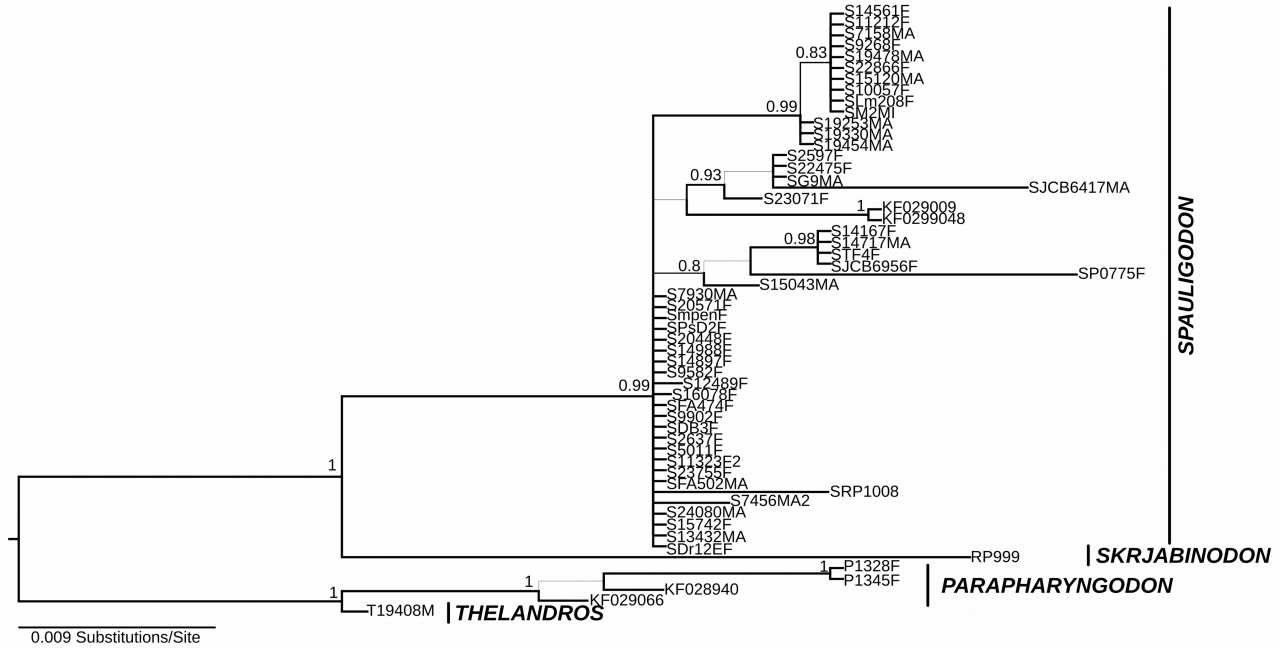

Fig.3.1- Bayesian 50% majority-rule inference tree for the 18S parasite dataset. Branch labels show posterior probabilities (values below 0.75 not shown) and branch width drawn according to the respective posterior probability support.

Relative rate and time estimates

The Bayesian MCMC inference runs converged efficiently on a posterior mean value for all parameters. The phylogenetic trees present a different topology from the one estimated for the combined dataset in MRBAYES, but overall it was better supported (Fig.3.3). The mean evolutionary rates estimated for two nuclear and mitochondrial (1st and 2nd codon position) markers had a very similar posterior mean (nuclear mean rate: 6.83×10^{-4} subs/site/Mya, mitochondrial mean rate: 6.58×10^{-4} subs/site/Mya). Estimated molecular substitution posterior means and 95% HPD rates for each terminal branch are depicted in Fig.3.3.

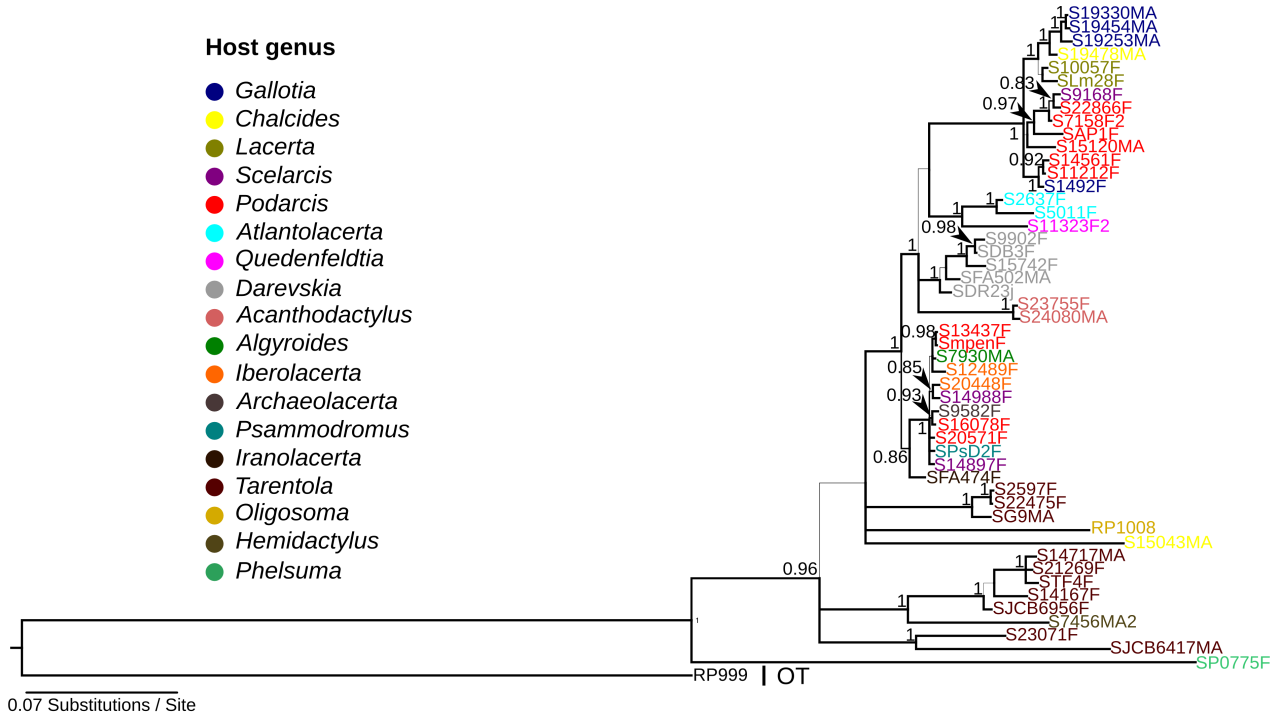

Fig.3.2- Bayesian 50% majority-rule inference tree for the concatenated ITS1, 28S and COI parasite dataset. Branch labels show posterior probabilities (values below 0.75 not shown) and branch width drawn according to the respective posterior probability support. Tip labels from each clade coloured according to the host genus. Outgroup (OT).

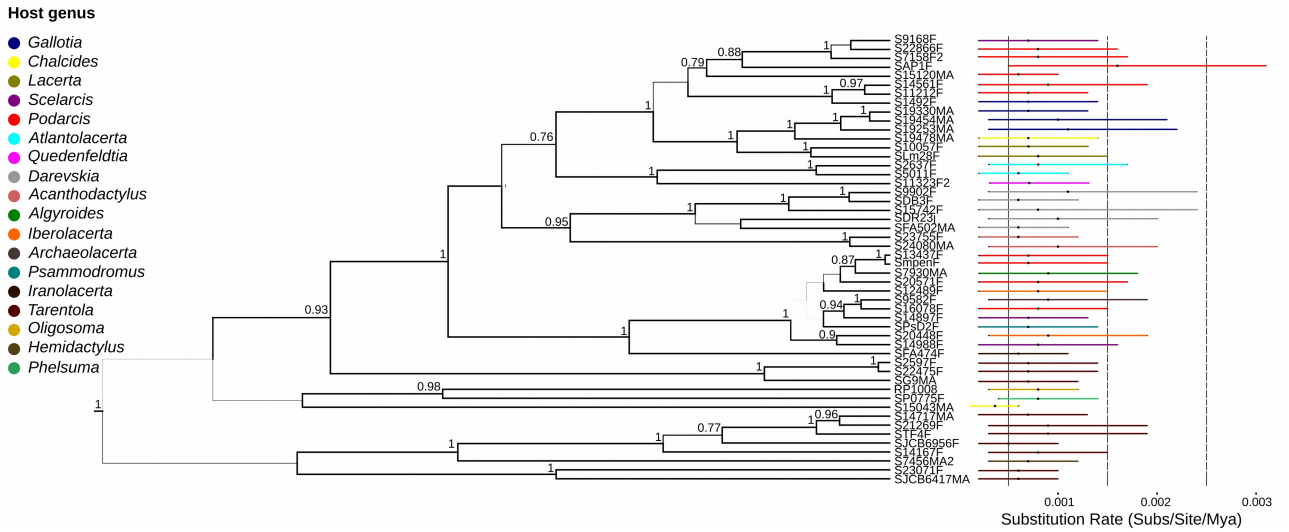

Fig.3.3- Maximum clade credibility ultrametric timescaled tree for the concatenated ITS1, 28S and COI parasite dataset with respective estimated posterior mean and 95% HPDs of the substitution rates. Node labels show mean divergence time estimates. Branch width drawn according to the respective posterior probability support. Rates 95% HPDs of each lineage coloured according to the host genus.

Host-Parasite coevolutionary history

The procrustes superimposition plot displayed in Fig.3.4 shows that several host-parasite associations have some degree of cophylogenetic structure. However, it also indicates that some associations do not represent a good fit between the parasite and host ordinations, as represented by the long arrows (Fig.3.4). The global goodness of fit provided evidence for overall significant

congruence between the parasite and host phylogenies (m^2 global value = 0.850, *P* = 0.00). The contribution of each individual host-parasite link to the procrustean fit is shown in Fig.3.5. We considered 22 of the 45 host-parasite links as congruent, whereas with the conservative approach only 8 were considered as congruent.

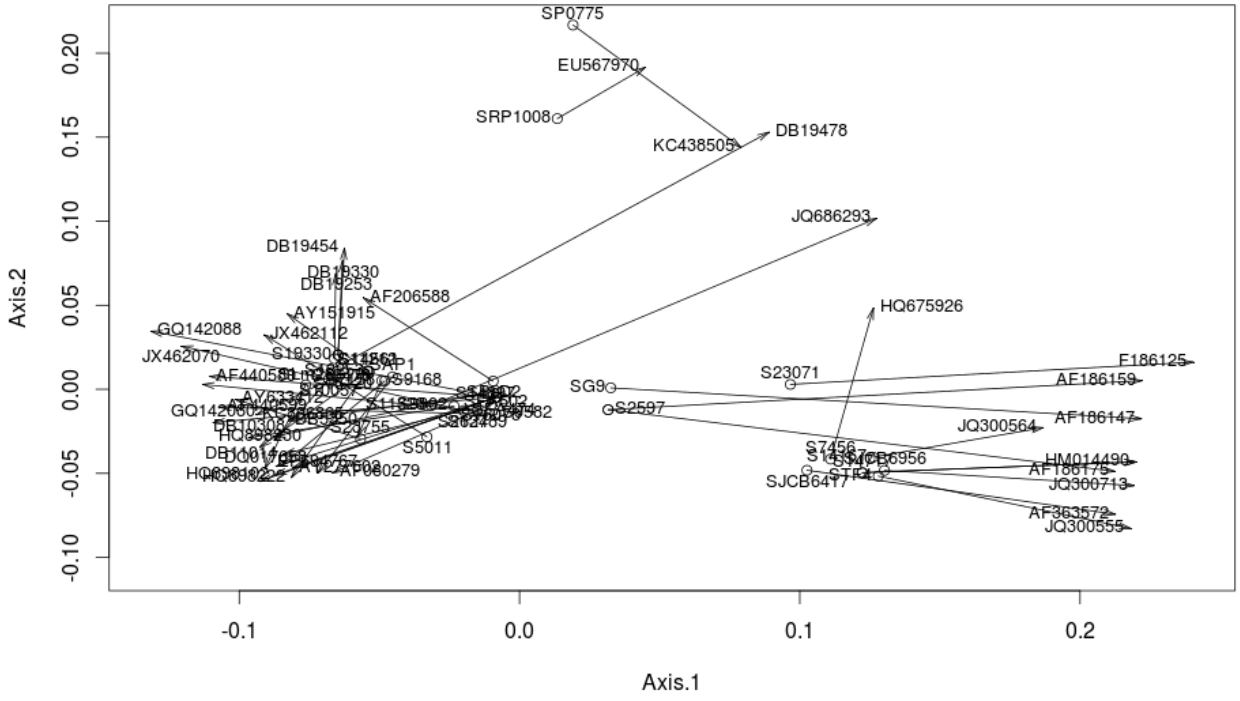

Fig.3.4- Procrustean superimposition plot for *Spauligodon* parasites and their respective hosts. Dots correspond to parasites and arrow tips to the hosts.

Rate comparisons

We did not detect a significant differences between the congruent and incongruent parasites lineages estimated mean rates for either of the employed classifications [mean approach: W = 314.5, *P* = 0.15; conservative (upper 95% confidence interval) approach: W = 144.5, *P* = 0.93]. A weak negative correlation was found between the squared residuals of each parasite lineage and the respective estimated mean rate [mean approach: rho = -0.29; conservative (upper 95% confidence interval) approach: rho = -0.22], as well as for the relative time of the host-parasite association (rho = -0.22), but without a significant value (all cases *P* > 0.05). The estimated mean rate was not determined by the degree of congruence, or by the time of divergence of each parasite lineage (all cases *P* > 0.05).

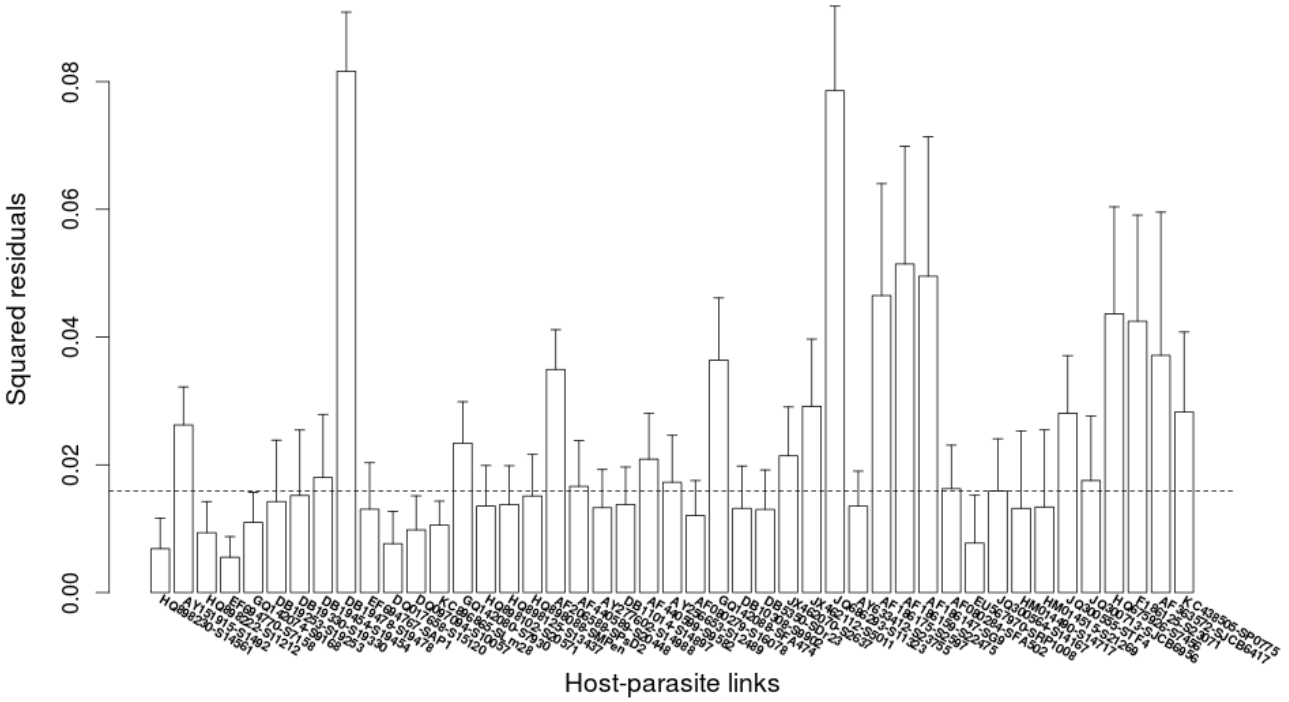

Fig.3.5- Contributions of individual host-parasite links to the Procrustean fit. Dashed line represents the median squared residual value.

Discussion

Our current knowledge of the underlying mechanisms causing different rates of molecular evolution is still limited and is mainly based on investigation of species biological traits. Parasites establish antagonistic interactions with their hosts, and such coevolutionary forces are likely to be responsible for rapid and divergent evolution, and a major driver of evolutionary change within species (Paterson et al. 2010). However, how different events, i.e. codivergence versus host-switches, influence parasite evolutionary rates, is still unclear. While demographic events, such as bottlenecks will have a whole genome-level effect (Charlesworth 2009), host-parasite antagonistic gene-by-gene interactions will have an effect on a select range of genes (Einer-Jensen et al. 2004; Paterson et al. 2010) and may or may not expand to the rest of the parasite's genome (Barrick and Lenski 2013). In this study, we hypothesised that different parasite coevolutionary histories would correlate with the rate of molecular evolution in neutral genes in *Spauligodon* parasitic nematodes. We found that the evolutionary history of these parasites does not mirror that of their hosts, despite an overall significant congruence of host and parasite phylogenies. In fact, several host switches are inferred to have occurred, even after acknowledging that the phylogeny of *Spauligodon* species is incomplete and some internal branches have low support (posterior probability < 0.75, Fig.3.2). All MCMC searches had evidence of convergence and proper mixing, indicating that the cause for

the low posterior values could be due to the size of our dataset, i.e. sequence length, and ultimately variability.

We tested if parasite lineages showing topology congruence with their ancestral hosts have an overall slower evolutionary rate, whereas those lineages resulting from switches to unrelated hosts have relatively higher rates. We found no evidence for a general correlation between the estimated coevolutionary parasite history (measured as the degree of congruence with the host phylogeny) and their respective estimated evolutionary rate. What we did find was that the measure of fit of the host-parasite association had a weak, negative correlation with the variation in the evolutionary rates, but it was not significant. This negative trend was not expected, and the explanation for it remains unclear. Nevertheless, no significant differences were found in the estimated rates between parasite lineages classified as congruent (as an approximation to codivergence) or incongruent (as a consequence of host-switch). Time is also an important factor to account for, since after a host-switch event, parasites may again establish a new “stable” and adapted association with their new host. We accounted for the divergence times for each parasite lineage as an estimate of the age of host-parasite association, but we found no evidence to suggest an influence of time on rates. The degree of congruence, the age of the host-parasite association, or their interaction were also not found to be a determinant of the evolutionary rates at neutral genes. Why did we not to find evidence for our initial hypothesis?

If rates do change:

In our approach we assumed that an increase in the evolutionary rate would leave a signature at the whole genome-level. As consequence, we expected that the rate changes would be observed in the nuclear and mitochondrial markers used in our study. Alternatively, even if a host switch event does increase the evolutionary rate, this is not influenced by demographic events (i.e. bottlenecks), but rather by new selective pressures on the parasite to out-evolve its host. Such pressures will only occur in genes directly involved in successful host exploitation, without any consequences or pressures in the other areas of the genome. This alternative hypothesis agrees with the observed pattern that rate variation in invertebrates is due to gene-by-lineage effects with adaptive substitutions forming a large component of the overall substitution rate (Thomas et al. 2006). If rates do change in *Spauligodon* parasites as a consequence of different coevolutionary scenarios, such influence may only be detected in a genome-wide analysis due to its effects in a selected range of genes. Regardless, time since the event can also be an important factor to account for in the understanding of parasite evolutionary rate. In fact all of the host-switch events identified in this system had occurred a long time ago, ranging from more than half a million years to few millions years ago. Therefore another possibility is that, even if rates did change after a successful host-switch event, other factors may have now erased the signature.

If rates do not change:

The evolutionary history of *Spauligodon* parasites seems to be ruled by the dynamics between host specificity and host shifts, reflecting a resilient plasticity of the parasites to expand their range by ecological fitting (Chapter 2). Our inability to find differences in evolutionary rates between congruent and incongruent lineages could just simply reflect that coevolutionary events do not influence rates in *Spauligodon* nematodes, not even when accounting for demographic effects. A new parasite association may not necessarily require extra effort for the parasite to re-establish their place in the arms race. In fact, the host-parasite interaction in this parasitic nematode may not fit the arms race model, thus the parasite does not need to run faster to adapt to a new host. Mutation is the ultimate source of the genetic variation required for adaptation, which is ultimately reflected in changes in rates of nucleotide substitution. However, overall parasites have higher mutation rates than their hosts, which in this case may be enough for the parasite to out-evolve its host without requiring an increase in its evolutionary rate.

Regardless of the points highlighted above we cannot rule out that those lineages, classified as incongruent by the global fit estimation, may indeed be in a stable coevolving stage with their respective host, and ultimately have decreased their evolutionary rates. This would also explain why we did not detect changes in rates. Additionally, we cannot discard that the sample size may have limited the power to detect any significant association. There is still limited knowledge of the variation in evolutionary rates in parasites, and unfortunately we could not enhance it with our study system. For now it will remain unclear if historical events along the evolutionary history of *Spauligodon* parasites, such as the two opposite events: codivergence and host switch, can influence rates of molecular evolution.

Conclusion

Unravelling how and why there is variation in evolutionary rates between species will ultimately allow us to understand the biology and evolution of an organism. However, this task is difficult and challenging due to complex relationships between underlying mutation rate dynamics, genomic architecture and selection. In host-parasite interactions, demographic events such as bottlenecks and/or pressures to out-evolve each antagonistic pattern can also interfere with the rate of molecular evolution, but it remains unclear if it happens in *Spauligodon* nematodes. Understanding the adaptability behind host shifts in terms of evolutionary changes for parasites is important not only to determine how new epidemics are generated, but also for understanding how host-parasite intimate associations evolve and culminate in species diversification.

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CHAPTER 4

Evolution of alternative male morphotypes in oxyurid nematodes: a case of convergence?

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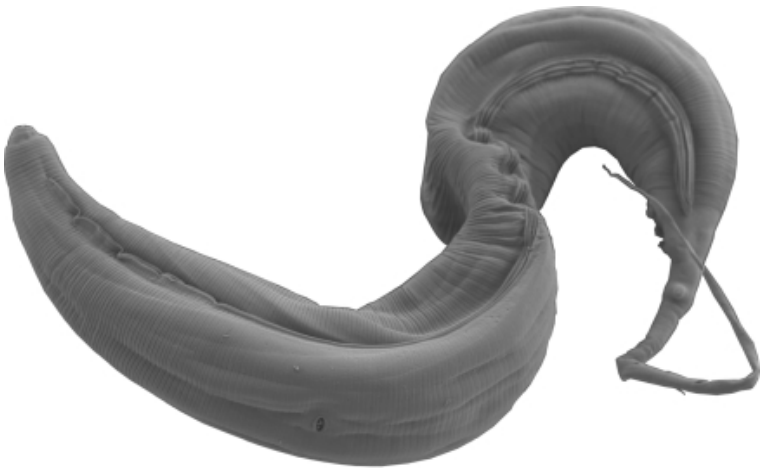

Spauligodon cabrerae, adult male

Abstract

Male dimorphism has been reported across different taxa and is usually expressed as the coexistence of a larger morph with exaggerated male traits and a smaller one with reduced traits. The evolution and maintenance of male dimorphism are still poorly understood for several of the species in which it has been observed. Here, we analyse male dimorphism in several species of reptile parasitic nematodes of the genus *Spauligodon*, in which a major male morph (exaggerated morph), which presents the traditional male morphological traits reported for this taxon, coexists with a minor morph with reduced morphological traits (i.e. reduced genital papillae) resembling more closely the males of the sister genus *Skrjabinodon* than *Spauligodon* major males. Because of the level of uncertainty in the results of ancestral state reconstruction, it is unclear if the existence of male dimorphism in this group represents independent instances of convergent evolution or an ancestral trait lost multiple times. Also, although the number of major males per host was positively correlated with the number of females, the same did not hold true for minor males, whose presence was not associated with any other ecological factor. Nevertheless, the existence of male dimorphism in *Spauligodon* nematodes is tentatively interpreted as resulting from alternative reproductive tactics, with differences in presence and number of individuals as indicators of differences in fitness, with the lower numbers of minor males per host likely maintained by negative frequency-dependent selection.

Introduction

Deviating from the general trend promoting a single morphology in each sex, there are several taxa where, rather than giving rise to a single fittest male and/or female phenotype, evolution has instead resulted in extreme phenotypic diversity (multiple adaptive peaks) with the existence of multiple morphs in the two sexes (Gross 1996). Alternative male phenotypes have been reported for a variety of species across different taxa, from invertebrates (i.e. arthropods: Buzatto et al. 2011; nematodes: Hoberg et al. 2012) to vertebrates (i.e. birds: Horton et al. 2012; fishes: Cogliati et al. 2013; reptiles: Calsbeek et al. 2010). The existence of alternative male phenotypes may result from alternative adaptation, in the broad sense (West-Eberhard 1986), or alternative reproductive tactics (ART), that is, conspecific, intrasexual competitors (Gross 1996; Tomkins and Hazel 2007; Oliveira et al. 2008). However, in both cases the underlying mechanisms that regulate phenotypic alternatives may be similar, and selection against intermediate phenotypes may lead to subsequent establishment of distinct intraspecific alternative forms (West-Eberhard 1986; Taborsky et al. 2008). In the first case, different adaptive phenotypes maintained in the same life stage and the same population may result from epigenetic divergence (i.e. heterochrony), giving rise to what West-Eberhard (1986) called intraspecific ‘alternative adaptations’. This allows a single species to

occupy more than one sympatric niche, thus increasing its adaptive potential (natural selection). Species with alternative strategies may also be less vulnerable to extinction and better able to adapt to new environments than monomorphic species (Pizzatto and Dubey 2012; Bastiaans et al. 2013). In the second case, evolution of male phenotypes might represent different solutions to reproductive competition (sexual selection), rather than a broader adaptive potential. ART are expected to arise whenever there is increased fitness to be gained by pursuing different reproductive tactics consisting in the specialization of same-sex conspecifics to exploit different reproductive niches, with their frequencies depending on the reproductive potential of each niche (Taborsky et al. 2008). Several theoretical frameworks around the strategies involved in ART have been proposed and the maintenance of alternative phenotypes has been widely discussed (see Gross 1996; Shuster and Wade 2003; Oliveira et al. 2008). However, most cases of ART probably result from both genes and environment contributing to the phenotypic expression of the different tactics (Brockmann 2001; Neff and Svensson 2013). The coexistence of multiple morphs, in the context of ART, is believed to be maintained through negative frequency-dependent selection (Iserbyt et al. 2013). Such alternative phenotypes are expected to persist in a population when their fitness curves cross, that is, when each does better than the other under some conditions (Brockmann 2001).

One of the most striking observations in male dimorphism is the convergence to similar general phenotypes shared across unrelated taxa usually involving allometric differences between several morphological traits, with a smaller male phenotype (or reduced morph, MI) and a larger one (or exaggerated morph, MA). Why are particular solutions so similar and frequent across a wide range of taxa? In the case of ART, this convergent selection may reflect a similar underlying process shaping its evolution across independent lineages (Mank and Avise 2006).

Given that in many cases taxonomy is still mainly based on morphological characters, the assessment of male dimorphism is of relevance in species description and has potential implications for taxonomy and systematics. This is especially true in groups such as parasitic nematodes, where genus diagnosis is frequently based on male morphology (i.e. Nematoda, Oxyurida: Ainsworth 1990; Rhabditida: Hoberg et al. 2012). For example, in oxyurid nematodes, Ainsworth (1990) identified male dimorphism in two species of *Skrjabinodon* nematodes, with morphological differences between male morphs consistent between the two species. Male dimorphism is also quite common in Ostertagiinae nematodes (Hoberg and Abrams 2001; Grillo et al. 2008; Hoberg et al. 2012). In one representative of this group, *Teladorsagia circumcincta*, the frequency of morphological polymorphism is primarily density dependent, with minor male morphs more likely to occur in high intensity infections, their intensity being positively correlated with that of the larger morph (Craig et al. 2010). However, differences in mating tactics of the different male morphotypes are still unknown among nematodes.

In this study, we combine genetic and ecological data to investigate the presence of male dimorphism across several species of the oxyurid nematode genus *Spauligodon* that infect reptiles. Specifically, we test the hypotheses that (i) similar patterns of male dimorphism have evolved convergently within different species and (ii) that the occurrence of the minor male morphs is frequency-dependent in natural infections as a possible consequence of ART. To test these two hypotheses, we build a phylogenetic framework to map the occurrence of dimorphic male morphotypes among *Spauligodon* species and statistically assess the role of ecological factors in driving the presence and extent of male dimorphism.

Material and Methods

Study system

Nematodes of the order Oxyurida are haplodiploid, that is, males derive from unfertilized eggs and are haploid, whereas females are diploid and develop from fertilized eggs (Adamson 1990). Within the order, members of the family Pharyngodonidae Travassos, 1919 are usually identified on the basis of male diagnostic morphological characters, females generally being very similar between groups. The main feature used to separate *Spauligodon* Skrjabin, Schikhobalova and Lagodovskaja, 1960 from other genera of the Pharyngodonidae family is primarily based on male morphology, namely the presence of caudal alae not supported by the last pair of genital papillae. The closely related genus, *Skrjabinodon* Inglis, 1968, is morphologically similar but lacks caudal alae, and their genital papillae are sessile and often reduced. Within the genus *Spauligodon*, species identification also relies on other male features, that is, shape of the caudal papillae and genital cone. Females are often indistinguishable between the two genera. In the Mediterranean and Macaronesian regions, the two genera are usually found in sympatry, infecting the same reptile host species (Roca et al. 1989, 2005; Hornero and Roca 1992).

Sampling procedures

A total of 916 samples, consisting of 593 intestines and 323 faecal pellets, were collected from 12 lizard species from different localities between 2009 and 2012 (Table 4.1; Data C.1, Supporting Information). Faecal samples were obtained either through spontaneous defecation of the reptiles when captured, or by gentle abdominal massage. Intestines were removed from dead animals, which accidentally died during fieldwork or that were euthanized through inhalation of ether vapours. All samples were preserved in 96% ethanol and examined for helminths, which were then separated, counted and identified. *Spauligodon* spp. specimens were mounted on temporary slides with a glycerol : water (1 : 1) solution and observed at different magnifications using a light microscope (Olympus CX41, Olympus Australia Pty Ltd, Notting Hill Victoria, Australia).

Representatives of each male morph from each species were genetically characterized. Prior to extraction specimens were photographed using a digital camera Olympus DP25 (Olympus®, Tokyo, Japan) and measured with the DP 2- BSW software (Olympus®).

Table 4.1 Prevalence (%), mean intensity (%), range and total number of individuals recovered of the two male morphotypes of each *Spauligodon* species, found in each type of sample, faeces and intestines.

Species	N Host	P MA	P MI	I MA	MA Range	I MI	MI Range	N MA	N MI	MA/MI
Intestines										
S. occidentalis	101	27.72	3.96	14.96	1 to 88	1	-	419	4	104.80
Spauligodon sp. PS*	2	0.00	50.00	0.00	-	1	-	0	1	0.00
S. carbonelli	20	70.00	5.00	7.71	1 to 21	1	-	108	1	108.00
S. saxicolae	397	2.27	4.28	1.44	1 to 3	1	-	13	17	0.76
Faeces										
S. occidentalis	106	26.42	7.55	6.04	1 to 30	1	-	169	8	21.13
S. atlanticus	105	21.90	3.81	5.91	1 to 27	1	1	136	4	34.00
Spauligodon sp. TG†	10	20.00	20.00	11.00	5 to 17	2	1 to 3	22	4	5.50
Spauligodon sp. PS*	83	7.23	1.59	2.67	1 to 9	1	-	16	1	16.00
S. carbonelli	19	0.00	0.00	0.00	-	0	-	0	0	-

MA, major male morphotype; MI, minor male morphotype; N Host, number of sampled hosts; P, Prevalence; I, Intensity; N MA, total number of MA; N MI, total number of MI; MA/MI, male morphs ratio (total number of MA/total number of MI).

**Spauligodon* sp. lineage infecting *Podarcis tiliguerta*.

†*Spauligodon* sp. lineage infecting *Tarentola gigas*.

Male morph discrimination

In all cases, two male morphs could be unambiguously identified based on several morphological features (Table 4.2, Fig.4.1). The major male morph (MA) corresponded to the phenotype typically associated with *Spauligodon* males, that is, presence of a caudal alae not supported by the last pair of genital papillae, genital cone present and genital papillae well developed and often pedunculated (Fig.4.1b, c). The minor male morph (MI) corresponded to males displaying the *Skrjabinodon* typical morphological features, that is, caudal alae and genital cone absent, genital papillae sessile and reduced, curled posterior end and spicule present (Table 4.2, Fig.4.1e, f). The MI was preliminary assigned to a particular *Spauligodon* species according to the respective conspecific MA males found in the same host individual, or within the same host population. In all cases, this assignation was later confirmed by genetic analyses.

DNA extraction and sequencing

Extraction of genomic DNA was performed on individual nematodes using the PureLink® Genomic DNA Kit (Invitrogen, Invitrogen New Zealand Ltd, Auckland, New Zealand) according to the manufacturer's protocol. To avoid cross-contamination, the two types of male morphs were extracted separately. Additional specimens from several *Spauligodon* species were also extracted (Table 4.3) to provide a more robust phylogenetic inference. Three partial gene fragments were

Table 4.2 Descriptive morphological data for the major (MA) and minor (MI) male morphotypes.

		S. occidentalis	*S. atlanticus*	*Spauligodon* sp. TG†	*Spauligodon* sp. PS*	*S. carbonelli*	*S. saxicolae*
BL MA		1402.61	1273.85	1065.68	689.00	1127.68	1027.74
Range (N)		982.07-1712.51(10)	1075.95-1497.96 (10)	843.06-1299.37 (10)	562.43-876.74 (10)	983.09-1169.59 (10)	635.55-1375.45 (8)
BL MI		944.39	739.64	742.17	762.79	1044.8	889.8
Range (N)		780.9-1125.55 (10)	557.93-898.74 (3)	728.88-752.56 (3)	758.84-766.74 (2)	- (1)	635.55-1144.64 (9)
BL MA/MI		1.49	1.72	1.44	0.90	1.08	1.16
BW MA		161.66	164.29	68.70	134.86	126.94	159.94
Range (N)		102.92-196.25(10)	134.42-180.93 (10)	62.16-75.64 (10)	103.22-188.99 (10)	103.94-163.96 (10)	89.85–194.55 (9)
BW MI		110.16	80.43	60.66	97.81	87.05	97.09
Range (N)		87.05-170.55 (10)	65.85-104.08 (3)	48.41-70.2 (3)	87.34 - 108.28 (2)	- (1)	67.11-120.51 (9)
BLW MA/MI		1.47	2.04	1.13	1.38	1.46	1.65
Spicule	MA					√	
	MI	√	√	√	√	√	√
Caudal alae	MA	√	√	√	√	√	√
	MI						
Genital cone	MA	√	√	√	√	√	√
	MI						
Genital papillae	MA	Ǫ	Ǫ	Ǫ	Ǫ	Ǫ	Ǫ
	MI	○	○	○	○	○	○

BL, Body length; BW, body width; √, presence; Ǫ, Pedunculated genital papillae; o, Reduced genital papillae*,

Spauligodon sp. lineage infecting *Tarentola gigas*†.

Spauligodon sp. lineage infecting *Podarcis tiliguerta.*

amplified: two nuclear genes, the 18S ribosomal RNA (18S) and 28S ribosomal RNA (28S) and one mitochondrial gene, the cytochrome oxidase subunit I (COI). The 18S was amplified using the primers Nem 18S F and Nem 18S R from Floyd et al. (2005). For the amplification of the 28S fragment, primers 28S rD1.2a and 28S B described by Whiting (2002) were used. The COI fragment was amplified using the nematode cocktail primers C_NemF1_t1 and C_NemR1_t1 from Prosser et al. (2013). Polymerase chain reactions (PCR) were performed in a total volume of 20 µL, comprising 4µL of MyTaq TM Red reaction buffer (Bioline, Bioline (Aust) Pty Ltd, Alexandria, NS W, Australia), primers at 0.5 mM each, 0.1 µL of MyTaq TM Red DNA Polymerase (Bioline) and 2–3 µL of extracted nematode DNA template. For all the sets of primers, PCR consisted of 35 iterations of the following cycle: 40 s at 95 °C, 40 s at 45–54 °C (depending on the primers used) and 1 min at 72 °C, beginning with an additional denaturation step of 3 min at 95 °C and ending with a final extension at 72 °C for 10 min. Amplified 18S and 28S fragments were sequenced for both strands with the same primers used in the amplification process, whereas for COI, following Prosser et al. (2013), the primers M13F and M13R (Messing 1993) were used. PCR product purification and sequencing was performed by a commercial facility (Macrogen Corporation, http://www.macrogen.com).

Phylogenetic analysis

The obtained sequences were imported into the software GENEIOUS v6.1.2 (Biomatters 2013) where contiguous sequences were assembled. Additional *Spauligodon* sequences published in previous studies were also included (Table 4.3). *Parapharyngodon echinatus* and *Thelandros tinerfensis* were used as outgroups for the 18S and 28S data sets, whereas *Spauligodon anolis* was used as an outgroup for the COI data set. Sequences where aligned with MAFFT v7.017 (Katoh et al. 2002) implemented in GENEIOUS v6.1.2 using the default parameters (auto algorithm; scoring matrix = 200 PAM/k = 2; gap open penalty = 1.53; and offset value = 0.123), followed by minor manual editing. For the COI, a total of 641 unambiguously aligned positions were obtained. The 18S and 28S alignments resulted in a total of 858 positions and 1133 positions including gaps, respectively. To determine the best fitting nucleotide model for the data set, the software JMODELTEST v2 (Darriba et al. 2012) was used under the Akaike Information Criterion (AIC). The models selected were: GTR+I, GTR+I+G and TIM3+I+G for the 18S, 28S and COI data sets, respectively. Phylogenetic analyses were performed using Bayesian inference (BI) and maximum likelihood (ML) methods, implementing the most appropriate parameters according to the estimated models. Bayesian analyses were performed in MRBAYES v3.2.1 (Huelsenbeck and Ronquist 2001) and ran for 10 x 10^6 generations with random starting trees, sampling every 100 generations. The first 25 000 trees were discarded as 'burn-in', after verifying that stationarity was reached by plotting log-likelihood values against generation time. A 50% majority-rule consensus tree was used to summarize the trees sampled from the post-burn-in trees. Maximum likelihood analyses were performed using PHYML v3.0 (Guindon and Gascuel 2003). Branch support was

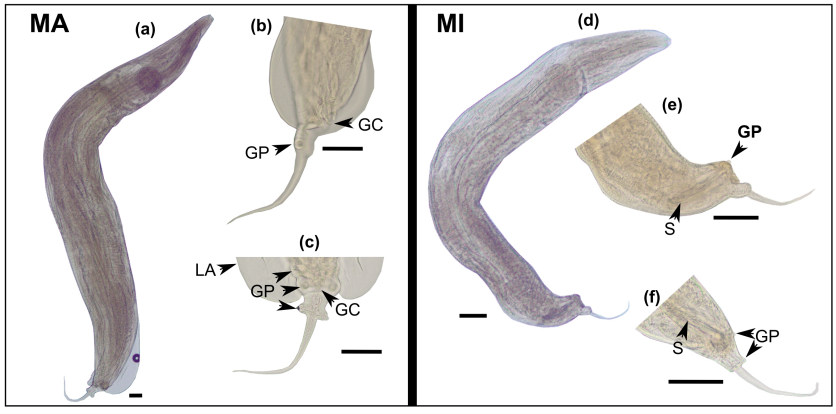

Fig.4.1- *Spauligodon* major (a–c) and minor (d–f) morphotypes. (a), general view of the major morph; (b), lateral view of major morph caudal extremity; (c), ventral view of major morph caudal extremity; (d), general view of the minor morph; (e); lateral view of minor morph caudal extremity; (f), ventral view of minor morph caudal extremity; MI, minor morph; MA, major morph. GP, genital papillae, GC, genital cone; S, spicule; LA, lateral alae. Scale 50 µm.

estimated by bootstrap analysis (Felsenstein 1985) with 1000 replicates. Additionally, to obtain a more robust inferred phylogeny to use in the ancestral state reconstruction (ASR), a BI was also performed for the combined data set of 28S and COI including only a single representative for

each species, using *P. echinatus* and *T. tinerfensis* as outgroups. Data were partitioned by gene, implementing the most appropriate parameters according to the respective estimated model for each partition. BI of the combined data was performed as described above. Estimates of pairwise uncorrected differences (*p*-distance) were made in MEGA v5 (Tamura et al. 2011). New sequences generated in this study were submitted to GenBank (Table 4.3).

Analysis of dimorphism evolution

The occurrence of male dimorphism was mapped on the inferred phylogeny based on the combined 28S and COI data, as a binary categorical trait: absent (0) or present (1). The evolution of male dimorphism was reconstructed under parsimony (MP) and likelihood (ML) based ancestral state reconstruction (ASR) approaches. Although MP minimizes the number of character state changes, ML methods consider every possible reconstruction, estimating the ancestral states that maximize the probability of the observed states evolving under a stochastic model (Cunningham et al. 1998). ASRs were performed using the ancestral state module implemented in MESQUITE v2.75 (Maddison and Maddison 2011) using the option 'trace character history'. The ancestral states were summarized on the 50% majority-rule consensus 28S and COI BI tree. We used the Markov k-state 1 (Mk1) model of evolution for the ML reconstructions, which assumes a single rate of transition between two character states, and in which any particular change is equally probable between the two-state character (presence or absence of male dimorphism). This model was implemented with the default settings (threshold when decisions made: 2.0). To facilitate comparison between MP and ML methods, both reconstructions were visualized under the 'Balls and Sticks' tree form. Although pies at the nodes represent relative likelihoods for ML reconstruction, in MP reconstruction pies only represent the estimated ancestral state.

Factors related to MI and MA presence and abundance

Our data set included the following variables: parasite species, host species, type of sample (intestines or faeces), number of hosts infected with the MA male morph, number of hosts infected with the MI male morph, number of MA male morphs per host, number of MI male morphs per host, number of females per host, sum of the two male morphs per host, operational sex ratio [total number of adult males/(total number of adult males + total number of adult females), following Kvarnemo and Ahnesjö 1996], total number of *Spauligodon* specimens per host, total number of other helminth species per host and total number of helminths per host. Parasitism was expressed at the host species level by calculating prevalence and mean intensity. Parasite prevalence was calculated as the ratio between the number of infected host individuals and the total number of sampled host individuals, and parasite mean intensity as the mean number of parasites per infected host. Given the differences in detectability and abundance of nematodes depending on the

origin of the samples [see Jorge et al. 2013 (Appendix A)], data retrieved from intestines and faecal samples were treated separately in further analyses. A Wilcoxon sign-rank test with continuity correction (function wilcoxon.test of the *R* package) was performed to determine if there

Table 4.3 Nematode specimens used in the phylogenetic analyses, including their respective host species, locality of origin, and GenBank accession numbers.

Species	Code	Host	Locality	Specimen	GenBank			Reference
					18S rDNA	28S rDNA	COI	
Spauligodon sp. TG	SjG4M2	*Tarentola gigas*	Raso Is., Cape Verde	MI	KJ778085	-	KJ778103	This study
Spauligodon sp. TG	SG9M	*Tarentola gigas*	Raso Is., Cape Verde	MA	KJ778086	KJ778095	KJ778104	This study
Spauligodon sp. TG	SG4F	*Tarentola gigas*	Raso Is., Cape Verde	F	KJ778088	KJ778096	KJ778105	This study
S. atlanticus	Sj16536	*Gallotia atlantica mahoratae*	Fuerteventura Is., Spain	MI	KJ778075	KJ778099	KJ778108	This study
S.occidentalis	Sj19454	*Gallotia caesaris caesaris*	El Hierro Is., Spain	MI	KJ778076	KJ778097	KJ778106	This study
S.occidentalis	S19454M	*Gallotia caesaris caesaris*	El Hierro Is., Spain	MA	KJ778077	KJ778098	KJ778107	This study
Spauligodon sp. PS	Sj15120	*Podarcis tiliguerta*	Sardinia Is., Italy	MI	KJ778078	-	KJ778110	This study
Spauligodon sp. PS	S15120M	*Podarcis tiliguerta*	Sardinia Is., Italy	MA	KJ778079	KJ778100	KJ778109	This study
S. saxicolae	SjDB3	*Darevskia bendimahiensis*	Turkey	MI	KJ778084	KJ778093	-	This study
S. saxicolae	DB3F	*Darevskia bendimahiensis*	Turkey	F	KJ778081	KJ778094	KJ778114	This study
S. saxicolae	SDR3KM	*Darevskia rudis*	Turkey	MA	-	-	KJ778112	This study
S. carbonelli	Sj13431	*Podarcis hispanica* PH2	Portugal	MI	KJ778083	KJ778091	-	This study
S. carbonelli	S13432M	*Podarcis hispanica* PH2	Portugal	MA	KJ778082	KJ778092	KJ778111	This study
S.auziensis		*Tarentola mauritanica*	Morocco	F	JF829225	JF829242	JF829264	Jorge et al. 2011
S. nicolauensis		*Tarentola bocagei*	São Nicolau Is., Cape Verde	F	JF829226	JF829243	JF829265	Jorge et al. 2011
S. nicolauensis	S2828M	*Tarentola nicolauensis*	São Nicolau Is., Cape Verde	F	KJ778087[a]	JN619358	JN619359	Jorge et al. 2012
S. cabrerae	S7414F	*Podarcis pityusensis*	Formentera Is., Spain	F	KJ778074	KJ778102	-	This study
S. cabrerae	SSA5F	*Podarcis lilfordi*	Menorca, Spain	F	-	KJ778101	KJ778113	This study
S. occidentalis		*Gallotia galloti galloti*	Tenerife Is., Spain	F	-	JF829256	JF829292	Jorge et al. 2011
S. occidentalis		*Gallotia caesaris caesaris*	El Hierro Is., Spain	F	JF829235	JF829261	JF829306	Jorge et al. 2011
S. occidentalis		*Gallotia caesaris caesaris*	El Hierro Is., Spain	F	-	JF829258	JF829303	Jorge et al. 2011
S. lacertae		*Lacerta strigata*	Armenia	F	JF829238	JF829252	JF829286	Jorge et al. 2011
S. lacertae		*Lacerta media*	Armenia	F	JF829237	JF829255	JF829287	Jorge et al. 2011
Spauligodon sp. PM		*Podarcis vaucheri*	Morocco	MA	JF829228	JF829247	JF829269	Jorge et al. 2011
Spauligodon sp. PM		*Podarcis hispanica* PHSS	SE, Spain	MA	JF829229	JF829248	JF829271	Jorge et al. 2011
S. atlanticus		*Gallotia atlantica atlantica*	Lanzarote Is., Spain	F	-	JF829250	JF829274	Jorge et al. 2011
S. atlanticus		*Gallotia atlantica mahoratae*	Fuerteventura Is., Spain	MA	-	-	JF829283	Jorge et al. 2011
S. atlanticus		*Gallotia atlantica mahoratae*	Fuerteventura Is., Spain	F	JF829230	JF829251	JF829285	Jorge et al. 2011
S. saxicolae		*Darevskia unisexualis*	Armenia	F	JF829227	JF829246	JF829266	Jorge et al. 2011
S. carbonelli	ShpenF	*Podarcis hispanica* PH1A	Portugal	F	KJ778080	KJ778090	-	This study
S. anolis		*Anolis* sp.	Puerto Rico		KF029009	-	KF029353	Falk&Perkins 2013
S. anolis		*Anolis* sp.	Puerto Rico		KF029048	KJ778089	KF029393	Falk&Perkins 2013
T. tinerfensis	Tt19408	*Tarentola gomerensis*	La Gomera Is., Spain		KJ778073	JF829241	-	This study
P. echinatus		*Gallotia atlantica mahoratae*	Fuerteventura Is., Spain		JF829224	JF829241	JF829263	Jorge et al. 2011
P. echinatus		*Gallotia atlantica mahoratae*	Fuerteventura Is., Spain		JF829223	JF829240	JF829262	Jorge et al. 2011

MI, minor male morphotype; MA, major male morphotype; F, female.

*Reference: this study

were differences in the presence and number of MI and MA per host, across all hosts. We further tested any correlation between number of MA morph and other variables using a nonparametric Spearman correlation (function rcorr, R package *Hmisc*, Harrell 2013). This test was not performed for the variable 'number of MI male morphs per host', as all host specimens analysed, with the exception of one, were infected with a single MI individual. Firstly, to test whether the presence of MI could be determined by the number of MA male morphs, we performed a generalized linear model with a logistic regression function using a binomial distribution with the presence or absence of MI morph as response variable and the number of MA as the predictor. Due to the low number of individuals infected in some host species (see Table 4.1), analyses were performed in pooled host

species. Secondly, to test the hypothesis that MI represents an alternative male strategy, we constructed a generalized linear mixed model fitting the presence of MI morph as response variable and operational sex ratio, number of females, number of other helminths and total number of helminths and the interaction between operational sex ratio and number of females as predictors. We controlled for variation between different host species by also including host identity as a random factor. The operational sex ratio is a good predictor of male contest competition for mates (Kvarnemo and Ahnesjö 1996; Shuster and Wade 2003). The total number of females and its interaction with operational sex ratio were included in the model to account for the possibility that the availability of females is more important than male competition. The number of other helminths and the total number of helminths were included as main factors to assess whether the presence of MI morph was related to the presence and number of other helminths. No other variables were included in the model to prevent over parameterization (see Burnham and Anderson 2002). All variables were standardized prior to analyses (standardize function, R package *arm*, Gelman et al. 2009). A full submodel set (including the null model) from the global model was created using the dredge function implemented in the *MuMIn* package (Bartoń 2009). Even considering the small size of our data set, we decided to employ information theoretic model averaging approach, a procedure that accounts for model selection uncertainty to obtain robust parameter estimates or predictions (Grueber et al. 2011). Model averaging was performed on the best submodels (ΔAICc < 4), obtained with the function get.models and after using the model.avg function from *MuMIn* R package (Bartoń 2009). As we aimed to determine which factors had the strongest effect on the response variable, we used the zero method for model averaging (Nakagawa and Frecklton 2010). To determine if the results obtained in our analyses were affected by the nature of the response variable (low presence of MI male morph) resulting in a large amount of absences, a post hoc resampling procedure was performed. Given the low reliability of the faecal samples for capturing the helminth communities in the intestine [Jorge et al. 2013 (Appendix A)], the analysis was conducted only for intestine samples. In each resampling, a sub-data set was generated consisting of the 24 samples where MI male morphs were found and an additional 24 random samples where this morph was absent. For each sub-data set, we used the model averaged from the best submodels (ΔAICc < 4) obtained in the previous analysis (i.e. a generalized linear mixed model fitting the presence of MI morph as response variable and operational sex ratio, number of females, number of other helminths and total number of helminths as predictors), implementing it as our new global model. We then performed a similar analysis as described above, with the exception that the new model average was performed over all the submodels. This procedure was repeated 1000 times with resampling without replacement over the absences data. All analyses were implemented using the package R version 3.0.2 (R Core Team 2013).

Results

Phylogenetic analyses

A total of 858 bp was used in the 18S analyses, whereas for the 28S and COI 1133 bp and 641 bp were used, respectively. For the combined COI and 28S data, 1774 bp was assembled. The 18S was the least informative marker with only 21 variable sites (29 including *Spauligodon anolis* sequences), against 154 for the 28S and 250 in COI (in all cases excluding respective outgroups). Nevertheless, all three markers retrieved the same pattern, with the MA and MI morphotypes clustering together irrespective of the species assigned (Fig.4.2 and 4.3), despite the unresolved topology between some branches. Estimates of sequence divergence between each pair of male morphotypes were never higher than 0% for the 18S, 0.1% for 28S or 0.8% for COI. We were not able to successfully amplify both 28S and COI markers for the two male morphotypes of *Spauligodon saxicolae*. However, the only MI amplified (SjDB3) clusters within the other specimens of *S. saxicolae* from Turkey, confirming its identification as a male morphotype of that species (Fig.4.3).

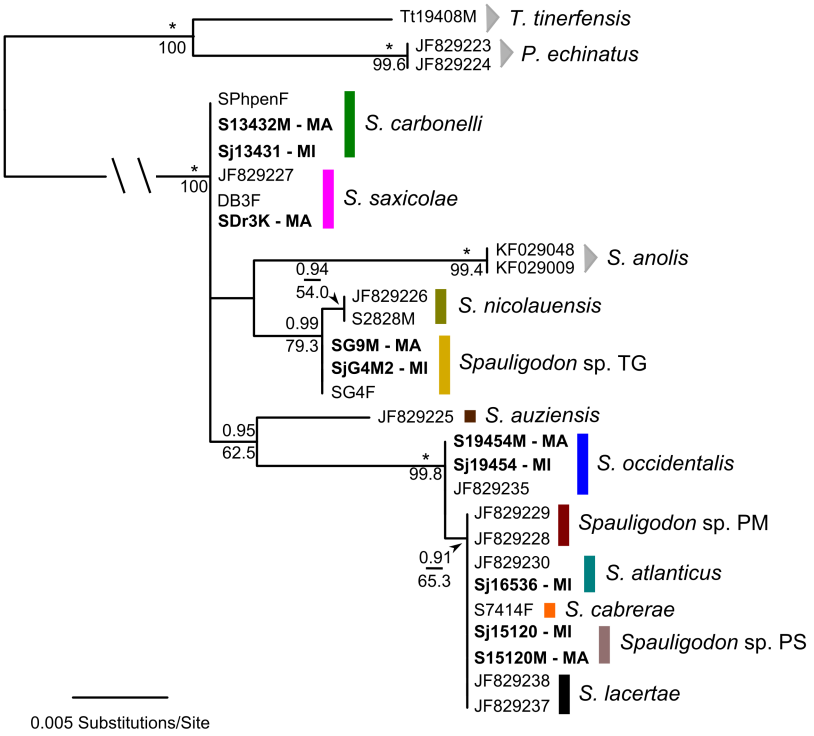

Fig.4.2- Maximum likelihood inference tree derived from 18S rRNA gene sequences. Values above branches represent Bayesian posterior probabilities and those below represent ML bootstrap support values (posterior probabilities below 0.75 and bootstrap values below 50 are not reported). *Corresponds to a posterior probability value of 1. Male morph specimens are in bold. For species details see Table 4.3.

Analysis of dimorphism evolution

The results from the ASR of male dimorphism in *Spauligodon* nematodes mapped over the 50% majority-rule consensus BI tree are represented in Fig.4.4. When ASR was performed under a

parsimony approach, presence of MI was retrieved as the ancestral state, whereas in the likelihood approach the reconstruction yielded 50% probability for each alternative state (ancestral vs. derived).

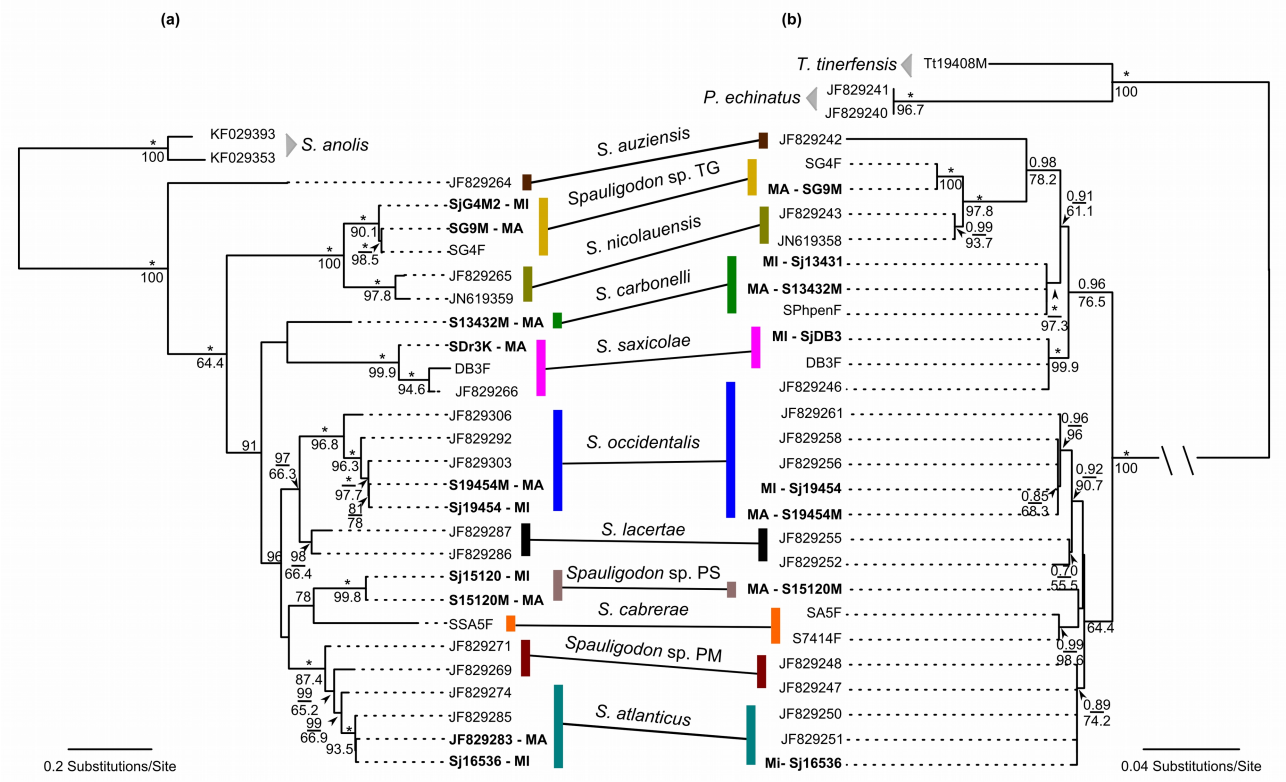

Fig.4.3- Maximum likelihood inference trees derived from cytochrome oxidase subunit I (a) and 28S rRNA (b) gene sequences. Values above branches represent Bayesian posterior probabilities and those below represent ML bootstrap support values (posterior probabilities below 0.75 and bootstrap values below 50 are not reported). *Corresponds to a posterior probability value of 1. Male morph specimens are in bold. For species details see Table 4.3.

Factors related to MI and MA presence and abundance

Prevalence and mean intensities of the two male morphs are presented in Table 4.1. The analysis of prevalence and intensity showed that the MA was significantly more prevalent than the MI [Wilcoxon sign-rank test with continuity correction; all pooled species: W = 1347.5, $P < 0.005$ in faecal samples (n = 64); W = 2223, $P < 0.005$ in intestine samples (n = 81)] and attained higher intensities (all pooled species: W = 1963.5, $P < 0.005$ in faecal samples; W = 2921, $P < 0.005$ in intestine samples). Although the number of MA per host ranged from 1 to 88 individuals, only one MI per host was found, with the exception of a single host that harboured three MI individuals (Table 4.1). The number of MA per host was positively correlated with the number of females (faeces: rho = 0.63, $P < 0.001$; intestines: rho = 0.55, $P < 0.001$) and the total number of helminths (faeces: rho = 0.4, $P = 0.0012$; intestines: rho = 0.78, $P < 0.001$), in both types of samples. Similarly, the total number of males was also positively correlated with number of females (faeces: rho = 0.6, $P < 0.001$; intestines: rho = 0.52, $P < 0.001$) and total number of helminths (faeces: rho =

0.42, $P < 0.001$; intestines: rho = 0.76, $P = 0$). Regarding the relationship between the two male morphs, there was a significant association between the intensity of MA and the presence of MI in faeces, but not in intestine samples (Faeces: $F_{1,62} = 7.021$, $P = 0.012$; intestines: $F_{1,79} = 1.407$, $P = 0.264$). The cut-off of 4 AICc yielded five and seven models for the presence of the MI morph among faecal and intestinal samples, respectively, which were then included in the model

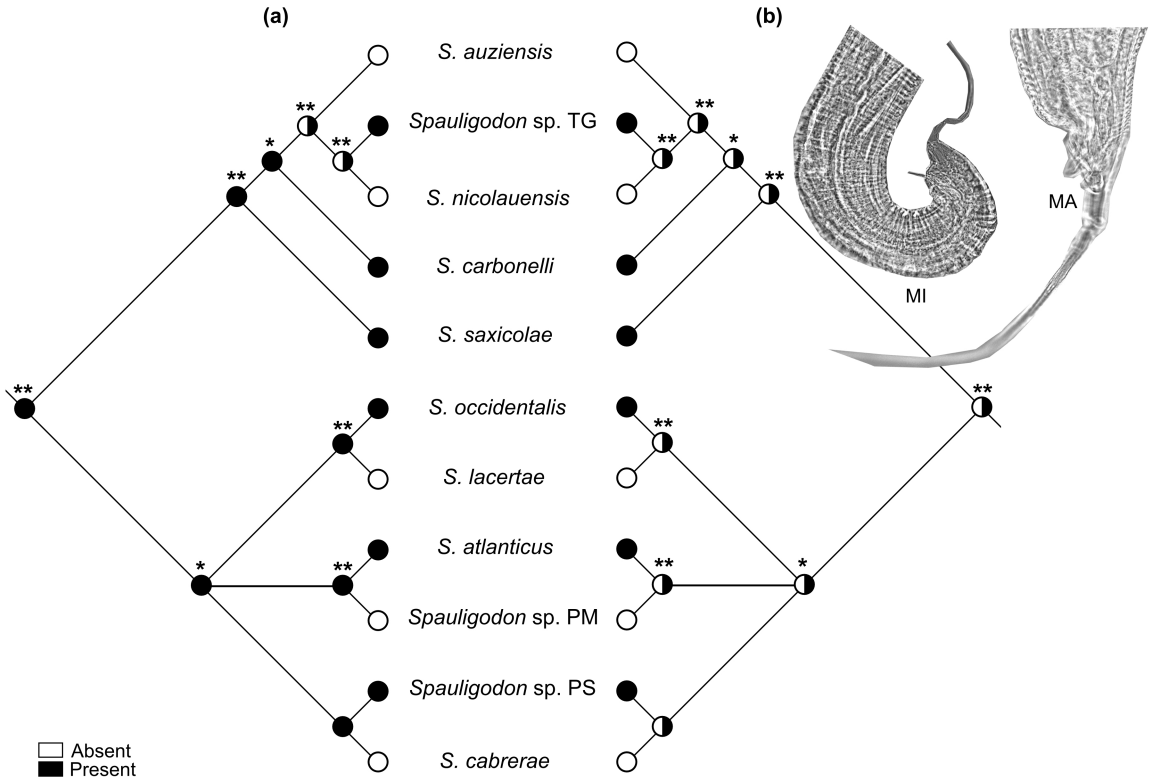

Fig.4.4- Ancestral state reconstructions for the *Spauligodon* male dimorphism based on parsimony (a) and likelihood (b), traced under 50% majority-rule consensus 28S and cytochrome oxidase subunit I Bayesian inference tree. Pie diagrams at each node: (a) parsimony reconstruction indicative of estimated ancestral state, (b) reconstruction indicate relative likelihoods for ML reconstruction; Asterisks denote Bayesian posterior probabilities values: *, 78–90%; **, 91–100%. For species details see Table 4.3.

Table 4.4 Summary results of the effects of several factors on the presence of the MI morph after model averaging. Analyses were performed separately for intestinal and faecal samples.

		Estimate*	Standard error	Confidence interval	Relative importance
Faeces	(Intercept)	-1.27	0.607	(-2.460,-0.080)	
	OSR	4.773	2.579	(1.437, 9.813)	1
	Fem	5.625	2.137	(-0.282, 9.827)	1
	Fem x OSR	5.085	3.347	(-1.475, 11.645)	0.84
	TI	-1.435	2.843	(-7.007, 4.138)	0.22
	Ospe	1.205	2.525	(-3.745, 6.153)	0.22
Intestines	(Intercept)	-1.023	0.424	(-1.854, -0.192)	
	OSR	0.255	0.549	(-0.820, 1.330)	0.18
	Fem	0.033	0.602	(-1.147, 1.214)	0.16
	TI	-0.1	0.776	(-1.622, 1.422)	0.17
	Ospe	-0.673	1.618	(-3.843, 2.498)	0.29

OSR, operational sex ratio; Fem, number of females; Fem x OSR, Fem and OSR interaction; TI, total number of helminths; Ospe, number of other non-*Spauligodon* helminths. *Effect sizes have been standardized on two SD following Gelman (2008).

averaging. For data collected from faeces, all parameters were included in the model averaging, but not for data collected from intestines, for which there was no interaction between number of females and operational sex ratio. The results from the model averaging after standardization (effect sizes are therefore on a comparable scale) are summarized in Table 4.4 From the faeces data, operational sex ratio and number of females were the most important predictors (100% relative importance) of MI presence, together with their interaction (84%). However, only the confidence intervals of OSR do not include zero and therefore only this factor is significant at a $\alpha = 0.05$. For the intestine data, no parameter was found to have an importance higher than 30% and all of their respective confidence intervals included zero. In the *post hoc* analysis, of the 1000 repetitions performed, we found significant results in only 12.3% of cases for the number of females per host, 6.9% for the total number of helminths per host, 1.4% for the operational sex ratio and 1.0% for the total number of other helminth species per host. The average relative importance of each of these predictors was never higher than 40% (total number of other helminth species per host: 40.99% ± 11.95; operational sex ratio: 32.98% ± 5.59;total number of helminths per host: 29.03% ± 4.06; number of females per host: 26.10% ± 2.13).

Discussion

In this study, we identified the existence of male dimorphism within six different *Spauligodon* species, characterized by a major (MA) and minor (MI) male morphotype. Despite the considerable morphological differences between them, morphs from the same host or within the same host population were phylogenetically related (Fig.4.2 and 4.3). In a previous study, Ainsworth (1990) reported the presence of male dimorphism also in the Pharyngodonidae family, in two *Skrjabinodon* species (*S. trimorphi* and *S. poicilandri*) infecting lizards from New Zealand, with a similar pattern: a smaller male morph (morph 2) which was found at lower intensity levels relative to the larger male morph (morph 1). In her study, both male morphs were haploid and did not present differences in the sperm cells, indicating that both were fertile. There are several common features shared between the minor morph found in *Skrjabinodon* spp. and those of *Spauligodon* reported in this study. These include overall smaller and thinner body size, reduction or absence of lateral alae, presence of a spicule, smaller aspinose tail, a curled posterior end and low intensities, almost never exceeding one individual per host. It is important to stress here that, even if the MI morph of *Spauligodon* species presents morphological characteristics best fitting the taxonomic description of *Skrjabinodon* males, this does not necessarily mean that the genus *Skrjabinodon* does not exist. Actually, preliminary results have shown that *Skrjabinodon poicilandri* from New Zealand is a sister taxa to *Spauligodo*n (unpublished data). From an evolutionary perspective, the maintenance of an overall similar male phenotype across different taxa may imply that these

features represent ancestral character states. In this case, contrary to the intrafamilial relationships proposed by Petter and Quentin (1976), *Skrjabinodon* should be considered plesiomorphic in the evolution of pharyngodonid nematodes that evolved in carnivorous hosts.

Due to the level of uncertainty in the results of ancestral state reconstruction, we cannot determine whether presence of the MI morph represents independent instances of evolution of similar traits due to convergence or parallelism, or if it constitutes a plesiomorphic trait that was lost multiple times. However, recurrent parallel forms may suggest ancestral developmental plasticity for producing both male morphs (West-Eberhard 2005). Additionally, the non-appearance of MI in other *Spauligodon* species may be a lack of detection resulting from low MI prevalence and intensity values rather than a true absence. Even within those species where the MI morph has been reported, we did not find it occurring in all sampled populations (e.g. in all sampled populations of *S. occidentalis* from the Canary Islands). In insects, male dimorphism has repeatedly evolved, and its maintenance is assumed to be dependent on spatial and temporal heterogeneity in the environment (Schwander and Leimar 2011).

Although the intensity of MA was positively associated with the intensities of females in the two types of samples, no significant association was found between MI and other variables in both types of samples. In oxyurid nematodes, males are produced parthenogenetically and typically are expelled in faeces before they become infective (Adamson 1990). Interestingly, auto-infective eggs have also been reported (Adamson 1990). Thus, in high intensities, females may be producing auto-infective haploid eggs which develop faster than females allowing offspring to mate with their mother (see Adamson 1990). The lack of agreement between the results collected from faeces and those from intestines makes it difficult to draw inferences on the factors driving the presence of MI morph and restricting their intensity per host. The operational sex ratio was found to be a good predictor for the presence of MI morph, but this was only valid for faeces and not for intestines. This discrepancy has been already discussed elsewhere [see Jorge et al. 2013 (Appendix A)], with intestines being the most reliable source of information for ecological studies of parasite associations. Therefore, relying only the intestine data, no variable was found to be a significant predictor of the presence of the MI morph. Consequently, we were not able to attribute the presence of this alternative male morph to any of the ecological variables investigated. We recognize that the present study is bound by several interpretational limitations which originate from the fact that the phenomenon is rare (only 24 samples of 593 intestinal samples and 15 of 323 faecal samples, contained MI males), thus resulting in a lack of power to detect any significant predictor. However, when the ratio between presence and absence of MI male morph was equalized in *post hoc* analyses, we still failed to find any variable to be a significantly important predictor of MI male morph presence. Significant effects were only detected in 1% to 14% of the repetitions, which could simply result from a random effect. This suggests that the lack of

sensitivity of the model might not simply be due to the rarity of the MI male morphotype, which indirectly suggests that, even by increasing our sample, we may not be able to change the outcome of our present analyses.

We cannot completely determine the nature of male dimorphism in these elusive parasitic nematodes. Male dimorphism could also result from a genetic disorder or other environmental and/or physiological factors. However, male dimorphism is common in different *Spauligodon* species, presenting the same low prevalence pattern. If this was a result from a genetic disorder, why would it be so common? It may instead be a case of ART, in which both morphs are the outcome, or are linked to, different reproductive strategies and contrasting mating tactics. In this case, we would expect that in some conditions the MI male morph would perform better than the MA male morph. Is the relative fitness of the MI morph higher at lower frequencies? Without an answer to that question, another explanation is that the phenomenon is a case of alternative adaptation, allowing the two male morphs to occupy more than one sympatric niche and thereby increasing the adaptive potential of the species, and having nothing to do with reproductive tactic. But why would the MI male morph be only present in such low numbers (only once exceeding one per host) if it occupies a niche different from that of the MA morph? To date, no developmental, physiological or behavioural experiments have been performed to test any of these possibilities. However, we tend to favour the hypothesis of ART for the following reasons: (I) physiologically, in both male morphs a brownish secretion located near the genital area was found, similar to that observed when MA morphs copulate with females (unpublished data), suggesting that the MI morph are also fertile. This is additionally supported with evidence from Ainsworth (1990) regarding fertility of *Skrjabinodon* male morphs, that is, both male morphs were haploid and did not present differences in the sperm cells, indicating that both were fertile. Fertility of the MI morph is a necessary condition for the ART hypothesis to be accepted. And (ii) ecologically, even if we did not find a good predictor for the presence of the MI morph, including male competition and overall helminths intensity, the characteristics of the MI (i.e. smaller size, reduced characters, lower intensity values in the host) resemble those reported in several examples of ART (see Oliveira et al. 2008). Regardless of its evolutionary origin, the presence of two morphs is generally interpreted in terms of difference in fitness. The lower prevalence and intensity of the MI morph suggest a lower fitness or, alternatively, that the tactic associated with the MI morph is only more successful when rare. In the latter scenario, the MI morph may be under a strong negative frequency-dependent selection, as observed in damselflies (Iserbyt et al. 2013), such that it is maintained at very low intensity levels. However, the presence of three MI individuals in a faecal sample is indicative that in some conditions this may be overcome. Then again, it is still unclear which are the fitness trade-offs of the two different male morphotypes and particularly how the MI morph is maintained in the population or what is its adaptive potential.

Understanding the evolution of alternative phenotypes and assessing how selection acts in decision making processes (Brockmann 2001; Oliveira et al. 2008) requires an integrative approach including developmental, physiological, morphological and behavioural studies. We admit that most questions regarding the origin of the two male morphotypes can only be answered by means of experimental procedures. However, given the parasitic nature and frequency of this system, and especially the lack of knowledge on physiology and development, the underlying mechanisms maintaining the MI morph remain unknown. Nevertheless, our findings will need to be replicated in the future on a taxonomically and numerically enlarged data set. Unfortunately, such additional sampling is currently not feasible due to the elusive nature of these nematodes; in particular, to increase our chances of detecting such a rare event, we would need to carry out invasive sampling (i.e. sacrifice the host). The presence of different morphotypes highlights once more the importance of integrating not only morphological characters into the analyses, but also other data sources, particularly genetic data. This is especially important for taxonomic groups for which investigating behaviour and physiology is intrinsically difficult and consequently groups that are characterized solely on morphological traits. If the understanding of alternative phenotypes is still far from complete in some well-studied groups (see Oliveira et al. 2008), the study of male dimorphism in parasites clearly lags much further behind.

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Supporting information

Additional Supporting Information can be found in the Appendix C.

Data C.1 Number of samples collected in this study from each host species.

Data deposited at Dryad: doi:10.5061/dryad.cb5f3

CHAPTER **5**

Cryptic species unveiled: the case of the nematode *Spauligodon atlanticus*

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Spauligodon occidentalis, adult male

Abstract

The implementation of molecular tools in parasitology has led to the discovery of numerous cryptic species. However, detailed morphological studies are needed to evaluate the cryptic nature of such species, as well as to provide an appropriate and formal description. Recent phylogenetic analyses using mitochondrial and nuclear genes have revealed that the nematode *Spauligodon atlanticus*, parasite of lizards of the genus *Gallotia* endemic to the Canary Islands, consists of two highly divergent and unrelated lineages, one in the eastern islands and the other in the western ones. This study provides a detailed morphological analysis of the two *S. atlanticus* lineages characterized genetically, based on body measurements and scanning electron microscopy. This integrative approach revealed phenotypic differences between them, despite their overall morphological resemblance. As a result, the new species *Spauligodon occidentalis* sp. nov., from the formerly western lineage, is described. The morphological similarity between the two *Spauligodon* species is better explained on the basis of evolutionary convergence, since both species parasitize *Gallotia* lizards. In addition to delimiting the new nematode species, this study highlights the importance of combining genetic and morphological data with taxonomy to uncover the nature of cryptic species and decrease taxonomic uncertainty.

Introduction

It is now relatively straightforward for taxonomists to incorporate multiple sources of information, including molecular and morphological, into a species description, an approach that strengthens both the empirical foundations of systematics and the Linnaean framework itself (Goldstein and DeSalle 2011). This integrative approach can provide taxonomists with a greater arsenal to face the realities of inventorying the actual biodiversity of the planet (Padial et al. 2010). Molecular tools offer an unprecedented opportunity to include new components in the discovery and description of biodiversity, merging contemporary technologies with the traditional morphological approaches (Nadler and Pérez-Ponce de León 2011). Such techniques are revealing a strong bias in the previous estimation of species richness, by identifying a significant number of cryptic species (Dobson et al. 2008). This is especially relevant in nematodes with microscopic structural differences, in which morphological assessment and identification of diagnostic characteristics are often difficult and require more technical and taxonomic expertise than those needed for macroscopic taxa (Abebe et al. 2011). Recently, several studies reported the discovery of cryptic species within what were considered single species, through the use of population genetics, phylogeographic or phylogenetic tools (Jorge et al. 2011; Nadler and Pérez-Ponce de León 2011; Poulin 2011a; Oliveira et al. 2012). Although several definitions of cryptic species exist (see Bickford et al. 2007; Nadler and Pérez-Ponce de León 2011), in the strict sense,

they can only be considered provisionally 'cryptic', since additional morphological studies or new high-resolution microscopy techniques may unveil diagnostic structural differences that allow a rapid and practical morphological diagnosis (Fritz et al. 2006; Pérez-Ponce de León and Nadler 2010). However, despite an increase in the number of species as a consequence of the implementation of molecular tools, further morphological studies providing an appropriate and formal description are often lacking. Consequently, there is an increase in taxonomic uncertainty that is counterproductive to research progress and synthesis in parasite systematics (Pérez-Ponce de León and Nadler 2010), considered by Littlewood (2011) as the 'cornerstone' of parasitology. Although other characteristics, such as host specificity, are relevant, morphology is still the primary source of data in parasite taxonomy, especially of metazoan parasites, although morphological characters may sometimes be misleading (Littlewood 2011; Perkins et al. 2011). Recently, there has been a dedicated effort to solve such problems in parasite systematics (Littlewood 2011) highlighting the advantages and disadvantages, promises and pitfalls of different approaches. It is now widely recognized that an integrative approach is needed to better assess parasite biodiversity, conciliating molecular tools with a traditional morphological approach that can be improved with high-resolution methods, including scanning electron microscopy and confocal microscopy. Although few such integrative studies on nematodes have been conducted (e.g. Fonseca et al. 2008; Falk et al. 2011; Razo-Mendivil and Pérez-Ponce de León 2011; Oliveira et al. 2012), they reinforce the importance and value of this approach, which should be more frequently implemented in parasitological studies.

One recent case of possible cryptic species in parasites has been observed in the nematode genus *Spauligodon*, infecting endemic lizards of the genus *Gallotia* in the Canarian archipelago (Jorge et al. 2011). *Spauligodon atlanticus* Astasio-Arbiza et al. 1987 was first described as a parasite of *Gallotia atlantica atlantica* Peters and Doria, 1882 and later identified in other host species of the same genus (Martin and Roca 2005). Despite the overall similar morphology, phylogenetic analysis revealed that *S. atlanticus* actually consists of two highly divergent lineages (12.9% uncorrected *p*-distance for COI). Moreover, the lineages are unrelated, suggesting that *Gallotia* spp. were colonized twice independently by *Spauligodon* nematodes (Jorge et al. 2011; but see Chapter 2). Given the clear polyphyly of S. *atlanticus* revealed by both mitochondrial and nuclear genes (cytochrome oxidase subunit I and 28S ribosomal RNA, respectively), Jorge et al. (2011) proposed the separation of the species, with the eastern lineage retaining the *S. atlanticus* designation, since the first description of the species was from *G. a. atlantica* from the easternmost island (Lanzarote), while the western lineage should be considered as a new species, which has not yet been formally described. In this study, we perform a detailed morphological analysis of the two *S. atlanticus* lineages, to determine the putative phenotypic differences between them and, if possible, to detect diagnostic characters. Subsequently, we formally describe a new species,

corresponding to the western lineage, and redescribe *Spauligodon atlanticus*, comparing previously phylogenetic evidences with morphological and morphometric characteristics obtained by means of light and scanning electron microscopy.

Material and Methods

Nematode isolation and vouchering

In 2009, nematodes of the genus *Spauligodon* were collected from six of the seven main islands of the Canarian archipelago (Fig.5.1) preserved in 96% ethanol and analysed phylogenetically by Jorge et al. (2011) (Fig.5.2). In this study, male and female specimens from the same localities and when possible from the same host specimen of the ones phylogenetically assessed were subjected to a detailed morphological analysis. Nematodes included in the previous

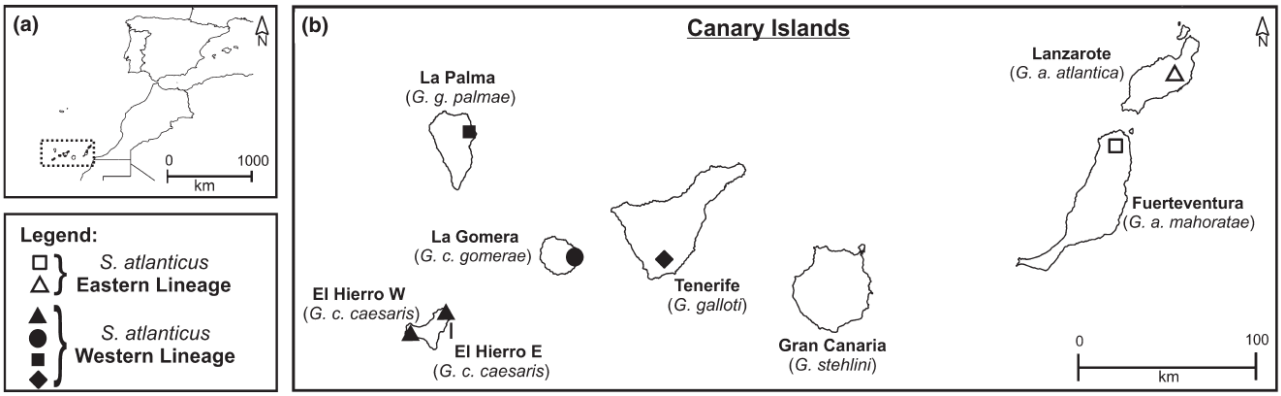

Fig.5.1- Map of the Canary Islands showing the geographical location of *Spauligodon atlanticus* samples included in the morphological analyses. a, geographical location of the Canary archipelago; b, Canary Islands.

genetic analysis (Jorge et al. 2011) could not be used in the morphological analysis due to limitations of the equipment used at the time. In addition, specimens collected in a previous expedition (Martin and Roca 2005) from the same localities and preserved in the same conditions (96% ethanol) were also included in the data set for morphological analysis. *Spauligodon* specimens were mounted on temporary slides with a bleaching solution (Foitová et al. 2008) and observed at different magnifications using a light microscope (Olympus CX41). All specimens were photographed using a digital camera Olympus DP25 and measured with the DP2-BSW software (Olympus®). Following De Ley et al. (2005), voucher videos were also assembled using several magnifications in different focal planes. Subsequent to the measurements, subsets of specimens were selected for scanning electron micrographs (SEM) and as vouchers to be deposited in museum (28 and 19, respectively). For scanning electron microscopy analysis, specimens were hydrated in an ethanol series followed by distilled water. They were then postfixed in 1% OsO_4, dehydrated through ethanol series and then dried to a critical point. The specimens were coated with AuPd to 10 nm thickness and examined with a Cambridge Instruments S460 scanning

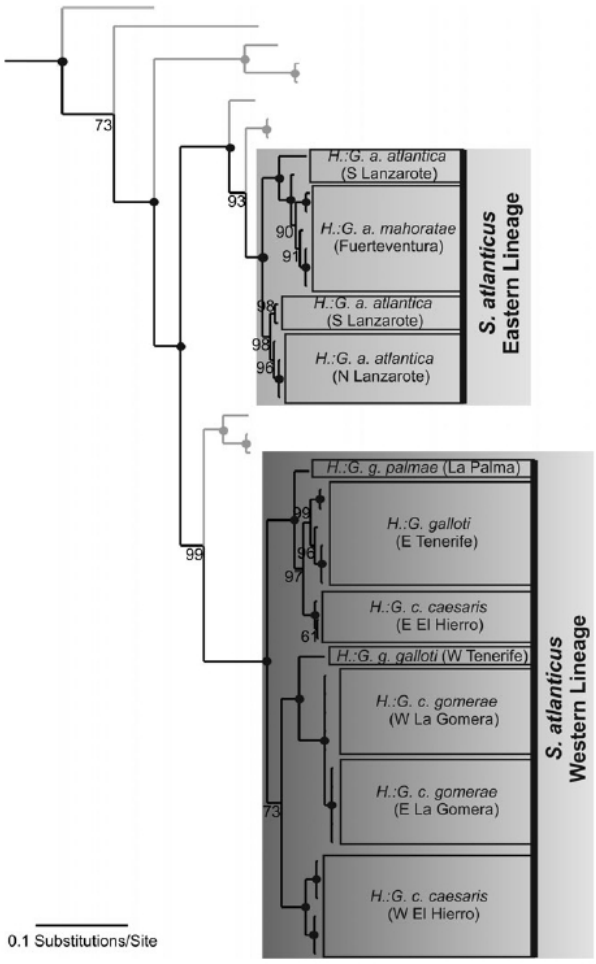

Fig.5.2.- Bayesian inference tree of the COI data for the *Spauligodon* spp. analysed in Jorge et al. (2011) with their respective host species (H) and localities. Values represent posterior probabilities. Bayesian clade credibility values of 100 are shown as a filled circle on the node. Adapted from Jorge et al. (2011).

electron microscope fitted with Dindima Image Slave frame grabber and with Zeiss Sigma VP FEG scanning electron microscope fitted with the HKL INCA Premium Synergy Integrated ED/EBSD system (the latter was only used for 10 specimens). Description photographs and videos have been deposited in MorphoBank (http://www.morphobank.org). Type vouchers and type specimens were deposited in the Natural History Museum, London. Additional specimens and DNA extractions are available upon request to the authors.

Morphology

Prior to the morphometric study, SEM were taken from fifteen *Spauligodon* specimens belonging to the two existing lineages (six specimens from the western and nine from the eastern lineage) in search of possible diagnostic morphometric characters. Preliminary micrographs suggested differentiation in the posterior region of the male, namely with regard to the size of the papillae. After this, seventeen characters in males (seven of them concerning the caudal extremity) and 14 in females were measured under a light microscope (Table 5.1, Fig.5.3), for a total of 63

Table 5.1. Descriptive statistics for all the linear measurements of adult specimens of the different lineages (eastern and western) taxa included in the multivariate analysis (in µm).

Eastern Lineage				
	Males (*N*=18)		Females (*N*=18)	
Character	Mean ± SD	Range	Mean ± SD	Range
BL	843.51 ± 119.01	659.4-1075.1	2846.45 ± 536.13	1835.28-3796.86
BW	136.19 ± 21.29	94.58-170.48	368.24 ± 60.43	273.35-473.09
OL	148.61 ± 19.04	106.04-183.34	261.57 ± 19.78	222.32-297.76
OW	23.46 ± 2.56	18.47-27.5	33.9 ± 4.27	25.1-41.76
OBL	54.09 ± 6.05	42.93-68.51	83.76 ± 8.52	63.42-96.11
OBW	58.92 ± 5.43	46.36-66.76	96.94 ± 7.29	84.14-111.46
NR	75.57 ± 14.68	40.42-99.17	107.45 ± 15.18	80.97-150
ExP	253.3 ± 31.88	205.2-328.44	235.34 ± 69.59	132.67-374.99
TL	219.89 ± 49.29	110.17-278.29	481.8 ± 31.41	430.08-539.26
LA	53.44 ± 11.38	39.44-68.39	-	
CT1	20.14 ± 1.55	17.64-22.8	-	
CT2	12.12 ± 1.26	9.25-13.82	-	
TW	12.84 ± 1.3	9.34-14.9	-	
3p1	5.39 ± 0.64	3.85-6.71	-	
3p2	4 ± 0.67	3.01-5.61	-	
3p3	8.67 ± 1.2	6.52-10.38	-	
3pl	8.33 ± 1.12	6.12-10.88	-	
Vu	-		282.56 ± 72.9	162.13-413.76
Va	-		448.14 ± 73.43	303.33-591.01
Weggm	-		36.12 ± 3.90	28.01-47.60
Leggm	-		121.35 ± 5.50	103.94-133.33
Spines	0		7.64 ± 1.03	6 to 9
Western lineage				
	Males (*N*=45)		Females (*N*=36)	
Character	Mean ± SD	Range	Mean ± SD	Range
BL	1317.45 ± 228.77	915.56-1749.14	3117.09 ± 535.11	2323.64-4457.37
BW	160.51 ± 28.68	98.93-219.02	382.6 ± 53.05	295.02-516.24
OL	264.04 ± 25.43	202.06-316.87	382.88 ± 47.51	156.41-449.3
OW	25.82 ± 3.04	20.13-32.83	35.93 ± 3.44	29.59-44.32
OBL	68.09 ± 9.23	49.61-90.1	103.04 ± 7.01	87.16-115.73
OBW	75.34 ± 10.15	51.96-98.21	117.05 ± 9.48	92.96-141.33
NR	119.75 ± 17.54	70.57-153.73	123.78 ± 10	104.01-142.97
ExP	395.65 ± 53.53	288.07-557.85	302.5 ± 67.29	200.08-417.03
TL	128.1 ± 16.95	98.95-165.66	389.7 ± 56.79	267.97-510.33
LA	93.12 ± 26.95	55.07-129.44	-	
CT1	30.59 ± 2.87	23.83-35.71	-	
CT2	16.58 ± 2.78	11.9-24.42	-	
TW	12.32 ± 1.26	10.21-15.3	-	
3p1	8.48 ± 1.1	5.25-11.03	-	
3p2	5.48 ± 1.24	2.22-8.06	-	
3p3	7.63-13.23	9.75 ± 1.2	-	
3pl	12.06 ± 1.54	9.14-14.95	-	
Vu	-		361.3 ± 70.33	239.28-475.61
Va	-		532.12 ± 73.21	390.63-727.45
Weggm	-		40.14 ± 3.49	33.71-51.45
Leggm	-		132.25 ± 6.41	115.97-152.27
Spines	0		7.31 ± 1.33	5 to 11

For each variable, mean ± standard deviation (SD), range and sample size (N) are given.

males and 54 females from seven localities. No females from La Palma were included in the analyses because of the limited number of specimens in good conditions. Representatives from all localities included in this study had been previously analysed genetically by Jorge et al. (2011) (Fig.5.1 and 5.2). All linear measurements (Fig.5.3) were recorded with the same equipment (camera/software/microscope) by the same person (FJ). Body length (BL) was measured from the

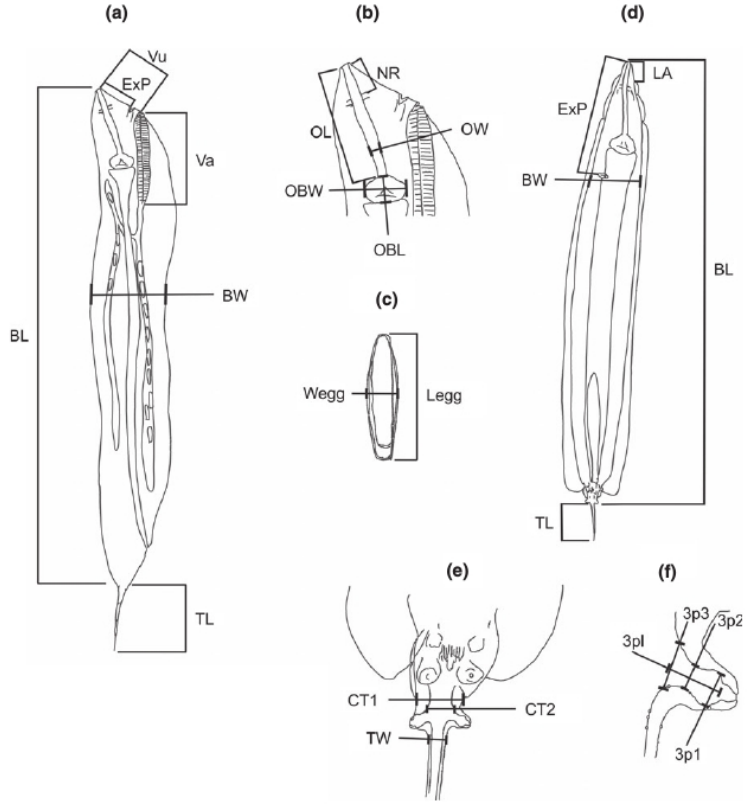

Fig.5.3- Linear measurements that were recorded for morphological analyses with their respective designation, for females (a-c) and males (d-f). See Material and methods for variables abbreviations.

anterior edge of the lips to the posterior edge of the body in females and to the posterior edge of the third pair of papillae in males. Body width (BW) was recorded at the middle body level in females and at the level of the excretory pore (excluding lateral alae) in males. Oesophagus length (OL) and width (OW) were recorded from the anterior border to the posterior margin that connects to the bulb, and at the level of the last third of the oesophagus, respectively. Length of oesophageal bulb (OBL) was recorded from the anterior border that connects with the oesophagus to the posterior margin, and oesophagus bulb width (OBW), at its widest point. Positions of the nerve ring (NR) and excretory pore (ExP) were recorded from the anterior edge to the nerve ring and excretory pore, respectively. Tail length (TL) was measured from insertion (at the base of the third pair of papillae in males) to its tip (broken tails were not included) and the tail width (TW) at its widest point. Lateral alae (LA) were measured from the anterior edge to the anterior beginning of the lateral alae (only measured in males). In males, measurements at the posterior end of the caudal papillae were also recorded (Fig.3e and f). Caudal trunk width was measured at its widest point (CT1) and narrowest point (CT2), at the insertion of the third pair of caudal papillae (Fig.5.3e). The width of one of the papilla of the third pair of caudal papillae was also measured, at the tip (3p1), middle (3p2) and insertion point (3p3). For the same papillae, the length (3pl) was also measured. Bilateral measurements were taken from the same side of the nematode whenever

possible. In females, vulva (Vu) position was also recorded from the anterior edge. Vagina (Va) was measured from the vulva until the posterior border of the vagina. Egg length (Legg) and egg width (Wegg) were also measured from the longest and widest parts, respectively, for a total of four eggs per female, and average egg length (Leggm) and width (Weggm) were calculated per individual. Spines were counted as the total number of cuticular spines present in tail.

Statistical analyses

Due to accentuated sexual dimorphism in *Spauligodon*, statistical analyses were conducted on males and females separately. Morphometric analyses were performed for a total of 117 specimens (63 males and 54 females; Data E.1, Supporting information), corresponding to seven localities (Fig.5.1) including 16 morphological variables in males (LA was not included) and 14 in females. Lateral alae (LA) were not included because data were missing for the majority of the specimens.

Prior to analysis, measurements were log-transformed and checked for homoscedasticity (Bartlett test) and normality (Shapiro–Wilk test) using the functions bartlett.test and shapiro.test of the *R* package, respectively (R Development Core Team 2011). Since several variables did not meet the assumptions, a nonparametric approach was followed.

To determine whether to include body length (BL) as a covariate in the subsequent analyses, nonparametric Spearman correlations between BL and remaining body measurements were performed, using the function cor of the *R* package (R Development Core Team 2011). Morphological differences between lineages were analysed using a permutational (multi)variate analysis of covariance (M) ANCOVA. This procedure is a good alternative to sum-of-squares-based (M) ANCOVAs, in cases where data do not meet normality and homoscedasticity assumptions (Anderson 2001). Permutational (M) ANCOVAs based on 1000 permutations were calculated using the function adonis implemented in the package *vegan* (Oksanen et al. 2012) of the R software (R Development Core Team 2011). A full model including the main effects of the factors LINEAGE, ISLAND considered as nested in LINEAGE, body length (BL) and their interaction was tested using a sequential sum of squares. The interaction factor was used to test the assumption of slope homogeneity (Engqvist 2005). Least squares means (LS means, adjusted means) and 95% confidence intervals were represented graphically using the software STATISTICA v7.1 (StatSoft Inc 2005).

To summarize the main sources of variation, principal component analysis (PCA) including all body measurements was performed for males and females separately. We used the *prcomp* function implemented in the package *R* (R Development Core Team 2011).

Results

Descriptive statistics of the biometric variables for all specimens are given in Table 5.1.

Males

BL was correlated with most of the body measurements, with the exception of TW (r = -0.13, *P* = 0.292). Correlations were positive in all cases, except in TL, which was negatively correlated with BL (r = -0.40, *P* < 0.001).

Body length (BL) and several other body measurements differed between lineages (Table 5.2, Fig.5.4). Regarding BL, individuals from the western *S. atlanticus* lineage were larger than the eastern ones (Fig.5.4). Specifically, males from El Hierro and La Palma (see Fig.5.1) were the largest, while the ones from Fuerteventura and Lanzarote were the smallest (Fig.5.4). However, despite the generally smaller size, individuals from the eastern lineage (Lanzarote and Fuerteventura) had comparatively longer tails (TL) (Table 5.2, Fig.5.4). Body width (BW) and tail width (TW) were similar in both lineages (Table 5.2, Fig.5.4). Caudal trunk (CT1 and CT2) was also larger in the western than in the eastern lineage (Table 5.2, Fig.5.4). Regarding the digestive tube, there were no differences in the oesophagus bulb size (nor length, OBL or width, OBW), although the western lineage had a longer (OL) but not wider (OW) oesophagus than the eastern lineage (Table 5.2, Fig.5.4). Both the nerve ring (NR) and excretory pore (ExP) were, in general, in a more posterior position in the western than in the eastern lineage (Table 5.2, Fig.5.4). Finally, the size of the third pair of caudal papillae also showed some differences; the western lineage had a larger and wider third pair of papillae (3pl and 3p1; see Fig.5.3 for details) but similar width of the peduncle (3p2 and 3p3; Table 5.2).

We also identified island differentiation within each lineage (Table 5.2, Fig.5.4). Within the eastern islands, individuals from Lanzarote were larger (BL) and had longer tails (TL), shorter oesophagus (OL), more anterior nerve ring position (NR) and wider insertion of the third papillae (3p3; Fig.5.4) than those from Fuerteventura. Regarding the western *S. atlanticus* lineage, individuals from La Palma and El Hierro were larger than the rest. In addition, specimens from La Palma were comparatively thinner (BW) and had a wider caudal trunk (CT2) and a wider base of the papillae (3p3) than the ones from the other western islands (Fig.5.4).

These differences were reflected in a good separation of the two lineages across the first two axes of the principal component analysis, encompassing 66.8% of the total morphological variance (Fig.5.5). The first component (PC1) explained 56.8% of the total variance; body size and most of the remaining variables related to body size have similar contribution and sign across the PC1, with the exception of tail length (TL) and tail width (TW) that have different signs (Table 5.3). PC1 showed a clear differentiation of the individuals from the eastern (Fuerteventura and Lanzarote) and western (remaining islands) lineages (Fig.5.5). The second principal component (PC2)

Table 5.2. Results of the permutational analysis of covariance on the males of *Spauligodon* showing the effects of the factors lineage, island nested in lineage, and their interaction, on body measurements using body length as covariate. For each variable, sequential sum of squares (SS), F statistic (F), R-squared values (R^2) and *P*-values (*P*) are shown. Significant results ($P < 0.05$) are in bold.

	BL				Lineage				Lineage:Island			
	SS	*F*	R^2	*P*	SS	*F*	R^2	*P*	SS	*F*	R^2	*P*
BL					0.470	192.173	0.587	**0.001**	0.191	19.627	0.239	**0.001**
MANCOVA	3.970	93.688	0.484	**0.001**	1.179	27.829	0.144	**0.001**	0.531	3.132	0.065	**0.004**
BW	0.140	54.423	0.329	**0.001**	0.003	1.275	0.008	0.264	0.133	12.986	0.314	**0.001**
OL	0.677	557.106	0.717	**0.001**	0.176	145.123	0.187	**0.001**	0.011	2.292	0.012	0.083
OW	0.069	40.569	0.394	**0.001**	0.007	3.998	0.039	0.057	0.004	0.528	0.021	0.718
OBL	0.195	92.778	0.618	**0.001**	0.001	0.258	0.002	0.599	0.001	0.068	0.002	0.989
OBW	0.205	115.359	0.623	**0.001**	0.002	1.045	0.006	0.302	0.002	0.211	0.005	0.943
NR	0.292	61.378	0.331	**0.001**	0.244	51.287	0.276	**0.001**	0.059	3.089	0.067	**0.031**
ExP	0.546	326.417	0.801	**0.001**	0.040	24.078	0.059	**0.001**	0.006	0.852	0.008	0.490
TL	0.333	77.394	0.324	**0.001**	0.330	76.692	0.321	**0.001**	0.127	7.386	0.124	**0.001**
CT1	0.343	328.406	0.667	**0.001**	0.097	92.575	0.188	**0.001**	0.008	1.832	0.015	0.131
CT2	0.231	75.380	0.474	**0.001**	0.028	9.019	0.057	**0.004**	0.043	3.472	0.087	**0.012**
TW	0.001	0.456	0.007	0.495	0.004	2.331	0.034	0.147	0.010	1.424	0.083	0.252
3p1	0.329	99.713	0.473	**0.001**	0.167	50.777	0.241	**0.001**	0.015	1.172	0.022	0.338
3p2	0.289	37.453	0.342	**0.001**	0.006	0.791	0.007	0.404	0.062	2.008	0.073	0.108
3p3	0.037	15.088	0.168	**0.002**	0.003	1.385	0.015	0.262	0.040	4.001	0.178	**0.006**
3pl	0.282	99.224	0.538	**0.001**	0.070	24.669	0.134	**0.001**	0.011	0.989	0.021	0.439
	BL*Lineage				BL*Lineage:Island							
	SS	*F*	R^2	*P*	SS	F	R^2	*P*				
BL												
MANCOVA	0.069	1.634	0.008	0.170	0.285	1.680	0.035	0.081				
BW	0.009	3.619	0.022	0.067	0.008	0.769	0.019	0.539				
OL	0.001	0.598	0.001	0.447	0.017	3.567	0.018	**0.011**				
OW	0.000	0.003	0.000	0.964	0.009	1.312	0.051	0.278				
OBL	0.010	4.854	0.032	**0.021**	0.002	0.259	0.007	0.904				
OBW	0.008	4.221	0.023	0.050	0.022	3.138	0.068	**0.027**				
NR	0.007	1.441	0.008	0.251	0.038	2.011	0.043	0.131				
ExP	0.000	0.009	0.000	0.923	0.005	0.704	0.007	0.586				
TL	0.006	1.298	0.005	0.266	0.012	0.698	0.012	0.602				
CT1	0.004	3.380	0.007	0.067	0.010	2.344	0.019	0.060				
CT2	0.019	6.288	0.040	**0.019**	0.010	0.827	0.021	0.509				
TW	0.001	0.344	0.005	0.548	0.015	2.124	0.124	0.088				
3p1	0.001	0.155	0.001	0.656	0.015	1.157	0.022	0.331				
3p2	0.000	0.009	0.000	0.938	0.094	3.053	0.112	**0.027**				
3p3	0.002	0.835	0.009	0.377	0.014	1.367	0.061	0.239				
3pl	0.003	1.037	0.006	0.317	0.013	1.100	0.024	0.380				

accounted for 10% of the total variation, with the variables contributing the most being body width (BW) and oesophagus width (OW) (Table 5.3). PC3 explained less than 7% of the variation. The variables contributing the most across this axis were 3p2 and 3p3 of the third pair of papillae (Table 5.3), although in this case, there was no clear differentiation of any specific population.

Females

All body measurements were correlated with body size (Spearman correlations, $P < 0.05$), with the exception of tail length (TL), number of spines in the tail (Spine) and egg width (Weggm) (in these cases, $P > 0.05$). In females, individuals from the western lineage were larger than those from the eastern one (BL, $P < 0.05$; Tables 5.1 and 5.4). However, as observed in males, western *S. atlanticus* had comparatively shorter tails than the eastern ones (Table 5.4, Fig.5.6), although

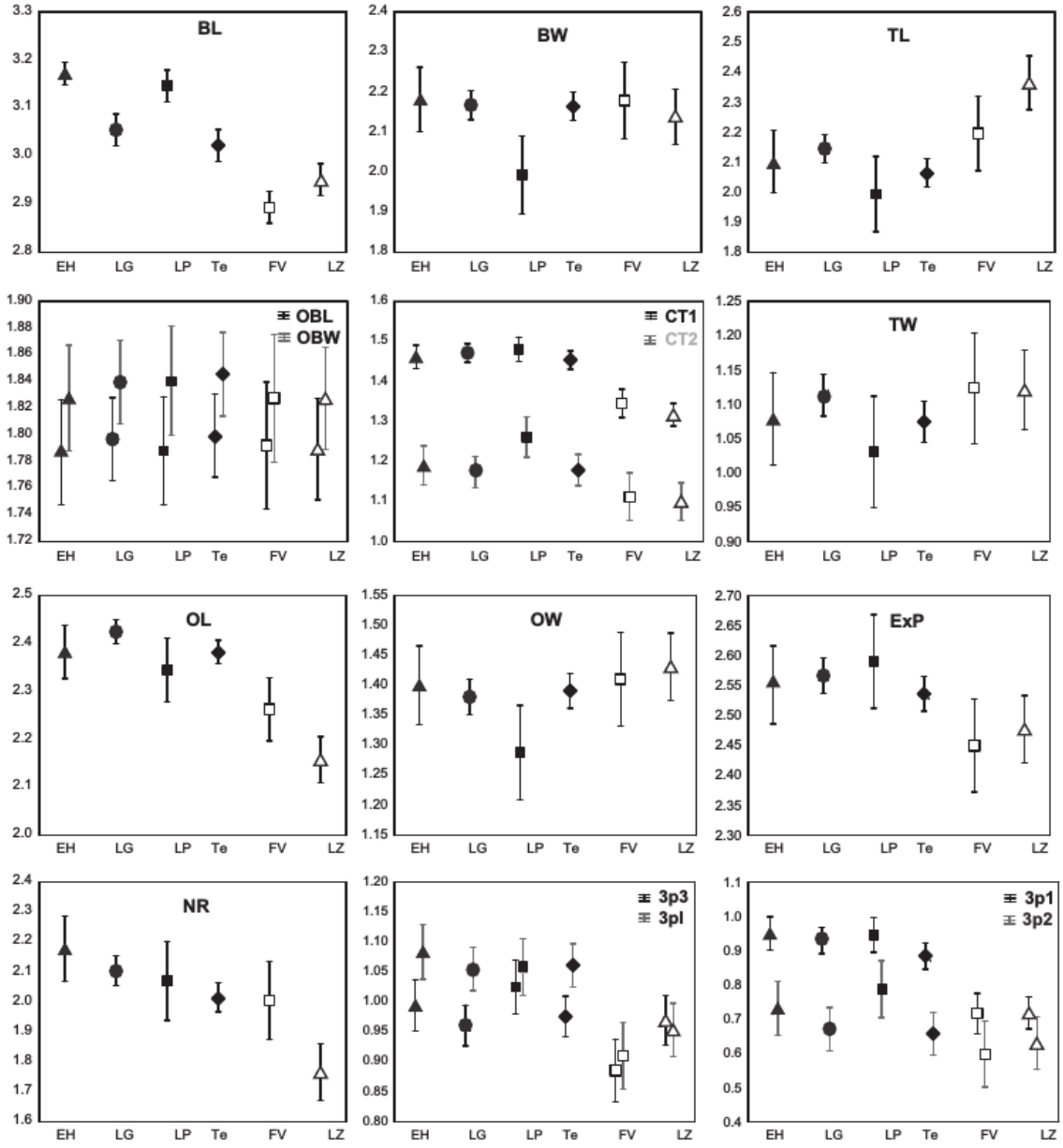

Fig.5.4- Representation of the adjusted means (Least square means, LS) and confidence intervals (95%) by island for all body measurements of *Spauligodon* male individuals included in this study. Mean body size (covariate) value used to compute males LS was 3.017. See Material and methods for variables abbreviations. Island abbreviations: El Hierro (EH), La Gomera (LG), La Palma (LP), Tenerife (Te), Fuerteventura (FV) and Lanzarote (LZ). Black symbols represent the western islands and white ones the eastern ones.

both had similar number of spines in the tail and similar body width (Table 5.4). Regarding the digestive system, there were no differences between lineages in oesophagus width (OW), although individuals from the western lineages had longer oesophagus (OL) and bigger oesophagus bulb (OBL and OBW, Table 5.4; Fig.5.6). The excretory pore (Exp), nerve ring (NR) and vulva (Vu) had, in general, a more posterior position in individuals from the western than in those from the eastern lineage. The vagina (Va) was also larger than in those from the eastern lineage. Regarding eggs, they were bigger (both in length and width) in the western lineage (Table 5.4; Fig.5.6).

Table 5.3 Variable loadings (eigenvalues) extracted from the three first principal components (PC) of the Principal Component Analysis (PCA) on males (left) and females (right). For each principal component, eigenvalues and % variance is shown.

	Males				Females		
	PC1	PC2	PC3		PC1	PC2	PC3
BL	0.306	-0.131	0.059	BL	0.249	0.431	-0.039
BW	0.182	-0.446	0.298	BW	0.210	0.429	-0.303
OL	0.305	0.078	0.241	OL	0.359	-0.236	-0.056
OW	0.189	-0.528	-0.119	OW	0.214	0.342	-0.049
OBL	0.271	-0.289	-0.063	OBL	0.374	-0.035	-0.087
OBW	0.277	-0.303	-0.148	OBW	0.331	-0.079	-0.331
NR	0.240	0.294	0.244	NR	0.280	-0.078	-0.072
ExP	0.298	0.017	0.118	ExP	0.295	0.085	0.554
TL	-0.234	-0.321	-0.150	TL	-0.171	0.423	0.095
CT1	0.309	0.087	0.102	Vu	0.304	0.099	0.518
CT2	0.254	0.216	-0.076	Va	0.297	0.094	-0.111
TW	-0.053	-0.001	0.356	Spines	-0.012	-0.012	0.424
3p1	0.280	0.215	-0.079	Leggm	0.249	-0.229	-0.023
3p2	0.221	0.133	-0.453	Weggm	0.183	-0.432	0.028
3p3	0.173	0.088	-0.584				
3pl	0.272	0.082	0.134				
Eigenvalues	9.095	1.600	1.101	Eigenvalues	5.863	2.280	1.278
%variance	56.850	10.000	6.880	%variance	41.880	16.280	9.130

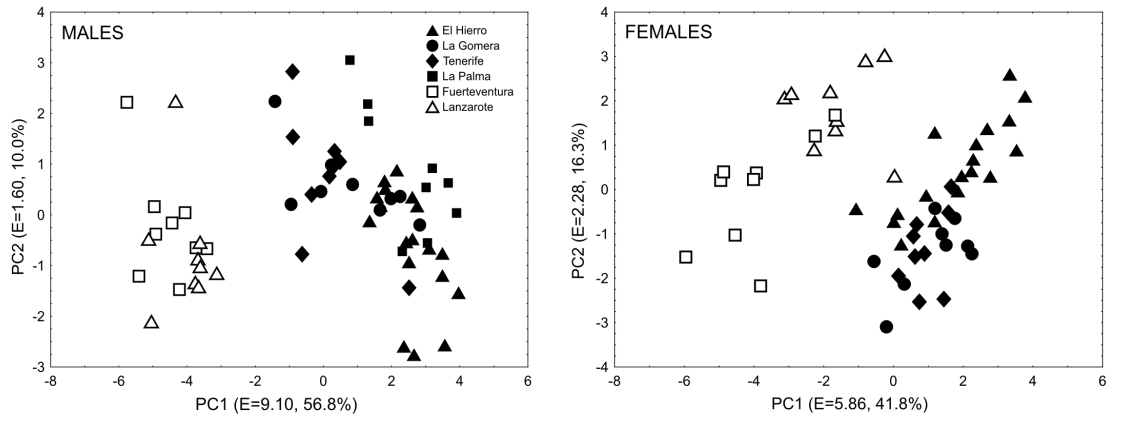

Fig.5.5- Representation of the distribution of the individuals across the first two principal component axes. For each axis, eigenvalues (E) and% contribution of each axis to the total variance are detailed.

We also found island variation within each lineage. Within the eastern lineage,individuals from Fuerteventura had a smaller body size (BL and BW) and digestive system (oesophagus length and width, OL, OW and oesophagus bulb length and width: OBL and OBW, Table 5.4, Fig.5.6) than individuals from Lanzarote. In addition, individuals from Fuerteventura had a more posterior excretory pore (ExP) and vulva (Vu) than those from Lanzarote. They also had a smaller vagina (Va), egg size, especially egg width (Weggm) and a lower number of spines in the tail (Spines; Fig.5.6). Regarding the western lineage, individuals from El Hierro were the largest, but had the shortest tails. They also had a smaller oesophagus bulb size (OBL, OBW). Individuals from La Gomera had the longest and thinnest digestive tubes (Table 5.4).

Table 5.4. Results of the permutational analysis of covariance on the females of *Spauligodon* showing the effects of the factors lineage, island nested in lineage and their interaction, on body measurements using body length as covariate. For each variable, sequential sum of squares (SS), F statistic (F), R-squared values (R2) and *P*-values (*P*) are shown. Significant results ($P < 0.05$) are in bold.

	BL				Lineage				Lineage:Island			
	SS	*F*	R^2	*P*	SS	*F*	R^2	*P*	SS	F	R^2	*P*
BL					0.020	7.712	0.062	**0.008**	0.179	22.597	0.544	**0.001**
MANCOVA	0.656	19.343	0.191	**0.001**	0.890	26.245	0.259	**0.001**	0.162	1.590	0.047	0.109
BW	0.105	51.995	0.486	**0.001**	0.000	0.157	0.001	0.705	0.007	1.129	0.032	0.337
OL	0.048	58.909	0.119	**0.001**	0.311	378.510	0.763	**0.001**	0.009	3.508	0.021	**0.025**
OW	0.037	25.917	0.300	**0.001**	0.002	1.513	0.018	0.228	0.016	3.842	0.134	**0.011**
OBL	0.039	46.934	0.232	**0.001**	0.076	91.350	0.451	**0.001**	0.013	5.115	0.076	**0.005**
OBW	0.016	18.737	0.114	**0.001**	0.067	76.289	0.466	**0.001**	0.011	4.139	0.076	**0.014**
NR	0.021	10.954	0.138	**0.002**	0.037	19.003	0.240	**0.001**	0.002	0.310	0.012	0.809
ExP	0.162	17.224	0.215	**0.001**	0.096	10.233	0.128	**0.001**	0.017	0.586	0.022	0.625
TL	0.007	3.680	0.029	0.061	0.122	65.778	0.514	**0.001**	0.018	3.239	0.076	**0.032**
Vu	0.151	22.127	0.244	**0.001**	0.090	13.231	0.146	**0.001**	0.013	0.653	0.022	0.592
Va	0.066	20.746	0.236	**0.001**	0.042	13.385	0.152	**0.001**	0.001	0.088	0.003	0.959
Weggm	0.001	1.319	0.016	0.269	0.031	31.598	0.385	**0.001**	0.001	0.405	0.015	0.723
Leggm	0.001	5.838	0.034	**0.023**	0.016	82.919	0.478	**0.001**	0.007	12.788	0.221	**0.001**
Spines	0.002	0.582	0.009	0.459	0.000	0.001	0.000	0.976	0.047	4.335	0.205	**0.009**
	BL*Lineage:Island				BL*Lineage							
	SS	*F*	R^2	*P*	SS	*F*	R^2	*P*				
BL												
MANCOVA	0.121	3.566	0.035	**0.014**	0.118	1.162	0.034	0.308				
BW	0.014	6.731	0.063	**0.011**	0.002	0.249	0.007	0.870				
OL	0.002	2.685	0.005	0.121	0.001	0.393	0.002	0.771				
OW	0.002	1.126	0.013	0.300	0.003	0.746	0.026	0.530				
OBL	0.000	0.001	0.000	0.975	0.004	1.580	0.023	0.215				
OBW	0.004	4.291	0.026	**0.045**	0.007	2.680	0.049	0.062				
NR	0.001	0.350	0.004	0.553	0.008	1.330	0.050	0.278				
ExP	0.029	3.129	0.039	0.088	0.036	1.267	0.047	0.299				
TL	0.004	2.084	0.016	0.186	0.005	0.905	0.021	0.441				
Vu	0.033	4.884	0.054	**0.023**	0.030	1.468	0.049	0.244				
Va	0.023	7.109	0.081	**0.013**	0.008	0.852	0.029	0.459				
Weggm	0.003	2.708	0.033	0.089	0.001	0.384	0.014	0.762				
Leggm	0.000	0.029	0.000	0.862	0.000	0.748	0.013	0.523				
Spines	0.007	2.036	0.032	0.155	0.014	1.238	0.059	0.296				

These morphometric differences were reflected in a good separation of the lineages in the multivariate analysis. The first principal component (PC1) explained 42% of the total variation. The variables contributing the most were body length (BL) and most of the other body measurements, all of them with similar contribution and positive sign in the first component, with the exception of tail length (TL) and to a lesser extent the number of spines (Spines) that also contributed to variation across the first axis, but with a negative sign (Table 5.3). These differences were responsible for the separation of the two lineages across the first axis (Fig.5.5). Regarding the second component (PC2), it explained 16% of the variation, with body length and width (BL and BW), oesophagus length and width (OL and OW), tail length (TL) and egg size (Weggm and

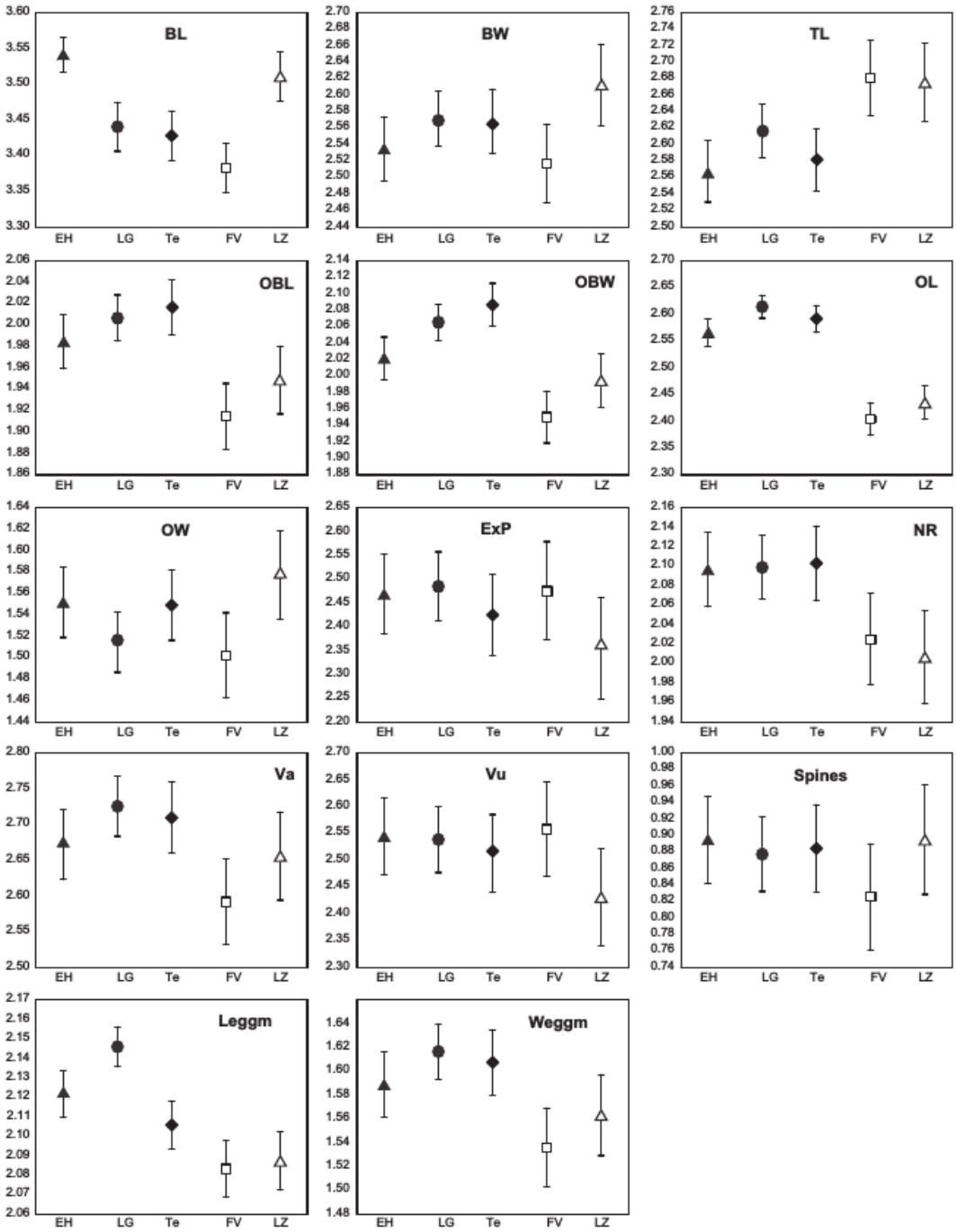

Fig.5.6- Representation of the adjusted means (Least square means, LS) and confidence intervals (95%) by island of all body measurements of *Spauligodon* females individuals included in this study. Mean body size (covariate) value used to compute females LS was 3.461. See Material and methods for variables abbreviations. Island abbreviations: El Hierro (EH), La Gomera (LG), La Palma (LP), Tenerife (Te), Fuerteventura (FV) and Lanzarote (LZ). Black symbols represent the western islands and white ones the eastern ones.

Leggm) being the most important variables (Table 5.3). Finally, the third component (PC3) explained 9% of the total variation, with body width (BW), oesophagus bulb width (OBW), position of the excretory pore (ExP) and the Vulva (Vu) and number of spines (Spine), being the most influential variables. PC3 did not separate clearly any population.

Integrative results and taxonomic summary

Altogether, the results presented here clearly demonstrate that the two genetic lineages retrieved by Jorge et al. (2011) are morphologically distinct and support their formal description as full species. In the subsequent paragraphs, the western lineage is described as a new species, whereas the eastern one is restricted to the original description of *S. atlanticus*.

Order Oxyurida Weinland, 1858
Family Pharyngodonidae Travassos, 1919
Genus *Spauligodon* Skrjabin, Schikhobalova and Lagodovskaja, 1960
Spauligodon occidentalis sp. nov.
MorphoBank M148036-M148105 and M148674-M148680
(Fig.5.7-5.8)

Diagnosis

Spauligodon occidentalis sp. nov. closely resembles *S. atlanticus* presenting on average larger males and females. Excretory pore and nerve ring in both males and females as well as vulva in females have a more posterior position. Females of the new species also possess a larger vagina. Males have a larger caudal extremity end (CT1 and CT2), and the third pair of caudal papillae consists of two large prominent papillae and larger peduncles. However, these morphological characters show overlap between the two species. The characters that unambiguously separate the two species are the molecular characters. *S. occidentalis* and *S. atlanticus* consisted of two genetically different, unrelated species, presenting 12.9% and 1.4% (uncorrected p-distance) of divergence for the COI and 28S rRNA, respectively.

Specimens examined

Eighty-one (45 males, 36 females; Table 5.1 and Data E.1, Supporting information).

Type material

Holotype: adult male (NHMUK 2012.9.13.1; MorphoBank M148057–M148058); Allotype: adult female (NHMUK 2012.9.13.2; MorphoBank M148055–M148056); and Paratypes: four males and four females (NHMUK 2012.9.13.3–10), from El Hierro Island (Canary Islands), Valverde (27.81798°N, 17.90859°W).

Etymology

The species epithet *occidentalis* alludes to the geographical distribution of the species, which is present in the western islands of the Canary archipelago.

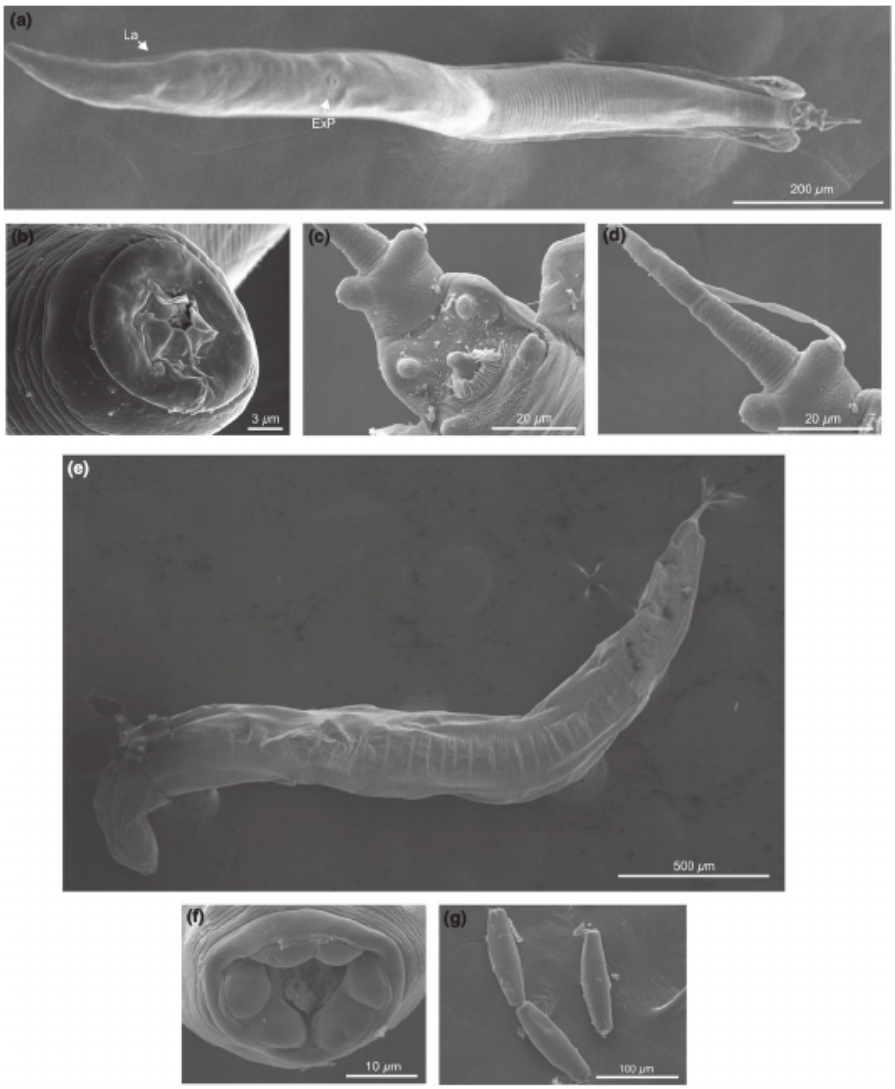

Fig.5.7- Scanning electron micrographs of *Spauligodon occidentalis* sp. nov. male (a–d) and female (e–g). a, general view of the male; b, cephalic end of the male; c, ventral view of the caudal extremity; d, ventral view of the third pair of caudal papillae; e, general view of the female; f, cephalic end of the female; g, eggs. La, lateral alae; ExP, excretory pore.

Description

Small-sized nematodes with cylindrical body with tapering anterior extremities and ending with a posterior filiform tail. Cuticle with distinct transversal striations more marked in males, starting after the lips and extending until the posterior extremity. Single lateral alae in males and discrete double lateral alae in females. Mouth opening triangular, enclosed by six labial plates in males and three bilobed lips in females. Short, straight oesophagus that ends in subspherical bulb.

Male: Small, filiform nematodes. Cuticle with distinct transversal striations, starting after the circumoral ring until the posterior extremity. Mouth opening triangular, enclosed by six equal overlapping labial plates, surrounded by a circumoral ring, which bears two amphids located on opposite sides. Excretory pore at the end of the first third of the body, surrounded by robust

cuticular ring. Very narrow lateral alae at its start, but progressively extending along the body, reaching its maximum width with auricular shape, projected on both sides of the cloaca. Three pairs of mammiliform caudal papillae, first two enclosed by caudal alae, third pair situated at the base of the tail directed outward, not enclosed by caudal alae. Precloacal pair (first pair) lies in higher area of posterior end, directed outward, consisting of two middle-sized spherical pedunculated papillae. Second pair, postanal, resembles first pair, but larger and elongated. Third pair consisting of two large prominent papillae, with thick, large peduncles. Genital cone situated in mid-ventral line, with an enlarged cuticularized proconus with double papillae and two lateral side pieces, surrounded by a pleated membranous curtain with coiled edges. Caudal extremity ends large and robust. Posterior end extending into aspinose, filiform tail. Spicule absent.

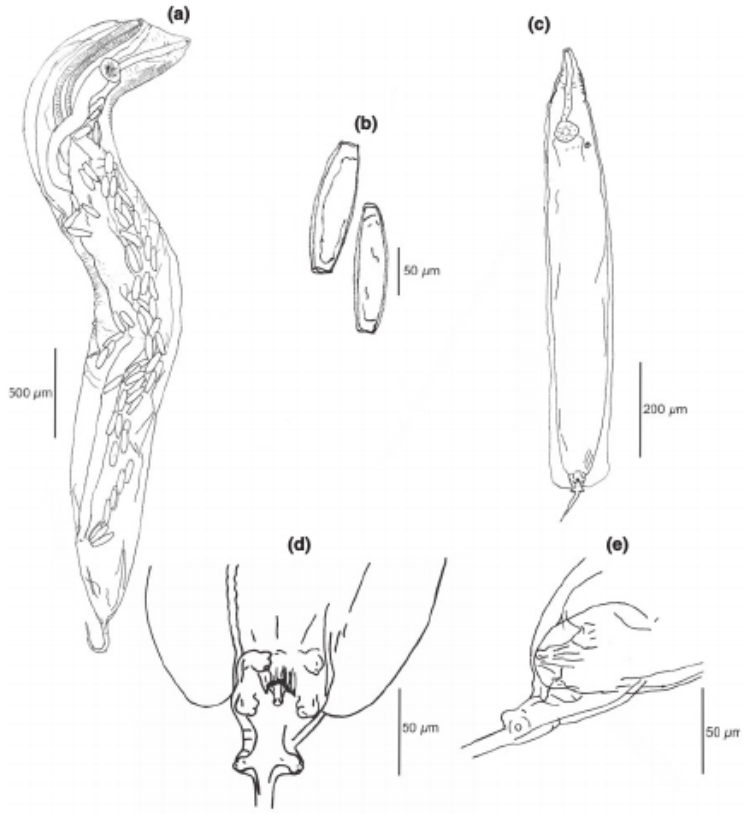

Fig.5.8- Drawings of female (a–b) and male (c–e) of *Spauligodon occidentalis* sp. nov. a, general view of the female; b, eggs; c, general view of the male; d, ventral view of the caudal extremity; e, lateral view of the caudal extremity.

Female: Filiform nematodes, larger than conspecific males (42% larger on average). Oral opening surrounded by three bilobed lips and with two opposite amphids. Cuticle with transversal striations, starting after the lips until the posterior extremity. Very tenuous, almost imperceptible double lateral alae, extending along the body. Excretory pore and vulva open at prebulbar level, surrounded by robust cuticular rings. Long, thick, muscular vagina, directed posteriorly. Ovaries located behind the vulva, females being opistodelphic. In fully gravid females, uterus extends anteriorly slightly past the vulva and posteriorly almost reaching the end of the body. Filiform tail,

with five to nine cuticular spines. Asymmetrical eggs, with truncated extremities and a polar cap in each pole.

Distribution

El Hierro, La Gomera, La Palma and Tenerife from Canary Islands, Spain.

Host species

This species has been identified from the intestine of the lizards *Gallotia caesaris caesaris* (Lehrs, 1914), *G. c. gomerae* (Boettger and Müller, 1914), *Gallotia galloti galloti* (Oudart, 1839), *G. g. eisentrauti* Bischoff, 1982 and *G. g. palmae* (Boettger and Müller, 1914).

Genetic and phylogeographic remarks

Spauligodon occidentalis sp. nov. is a highly divergent clade from *Spauligodon atlanticus* (12.9% uncorrected *p*-distance for the COI, Jorge et al. 2011; Fig.5.2). Although these species were previously considered conspecific, they apparently are not sister taxa (Jorge et al. 2011). *Spauligodon occidentalis* sp. nov. appears more closely related to *S. lacertae* identified in lizards from the subfamily Lacertinae than to *S. atlanticus*. *Spauligodon occidentalis* sp. nov. harbours greater genetic diversity than *S. atlanticus* (5.5% versus 2.7% uncorrected *p*-distance for COI, respectively; Jorge et al. 2011). The new species is present in the western, more recent islands of the Canarian archipelago (see Distribution). GenBank accession numbers: JF829231, JF829233-JF829235 (18S rRNA), JF829256–JF829261 (28S rRNA), JF829289–JF829315, and KC588965 (COI).

Order Oxyurida Weinland, 1858
Family Pharyngodonidae Travassos, 1919
Genus *Spauligodon* Skrjabin, Schikhobalova and Lagodovskaja, 1960
Spauligodon atlanticus Astasio-Arbiza, Zapatero-Ramos, Ojeda-Rosas and Solera-Puertas, 1987.
MorphoBank M148005-M148035 and M148671-M148673
(Fig.5.9–5.10)

Diagnosis

Spauligodon atlanticus is morphologically similar to *S. occidentalis* sp. nov. but has overall smaller size, except for the tail, which is larger (Table 5.1). In males, the third pair of caudal papillae is smaller with a thinner tip and a shorter peduncle (Table 5.1). Females with well-defined double lateral alae. However, the majority of the morphological characters show overlap between the two species. The characters that unambiguously separate the two species are the molecular

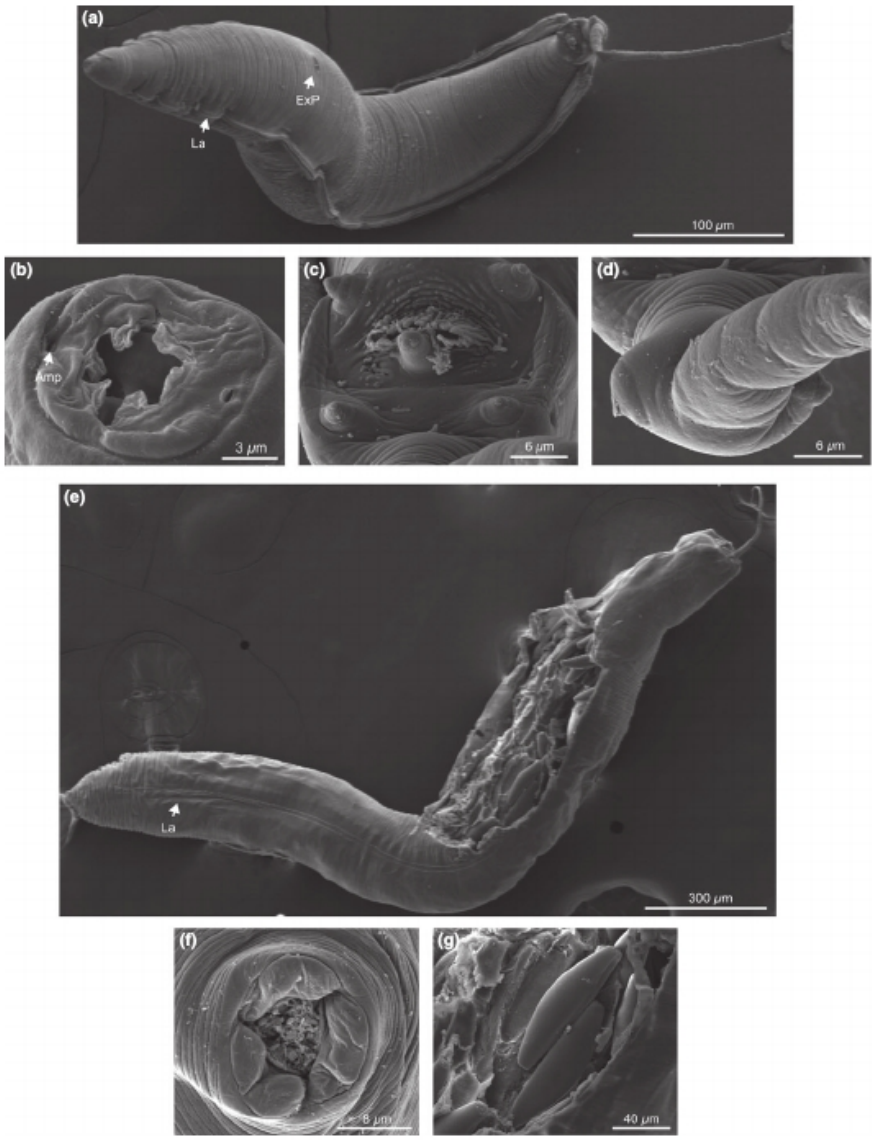

Fig.5.9- Scanning electron micrographs of *Spauligodon atlanticus* male (a-d) and female (e - g). a, general view of the male; b, cephalic end of the male; c, ventral view of the caudal extremity; d, ventral view of the third pair of caudal papillae; e, general view of the female; f, cephalic end of the female; g, eggs. La, lateral alae; ExP, excretory pore; Amp, amphid.

characters (see Diagnosis and Genetic and phylogeographic remarks of *S. occidentalis*).

Specimens examined

Thirty-six (18 males, 18 females; Table 5.1 and Data D.1, Supporting information).

Type material

Vouchers: four males and five females (NHMUK 2012.9.13.11–19; MorphoBank M148021–M148024, for only two of the males), from Lanzarote Island (Canary Islands), Nazaret (29.04646°N, 13.56206°W).

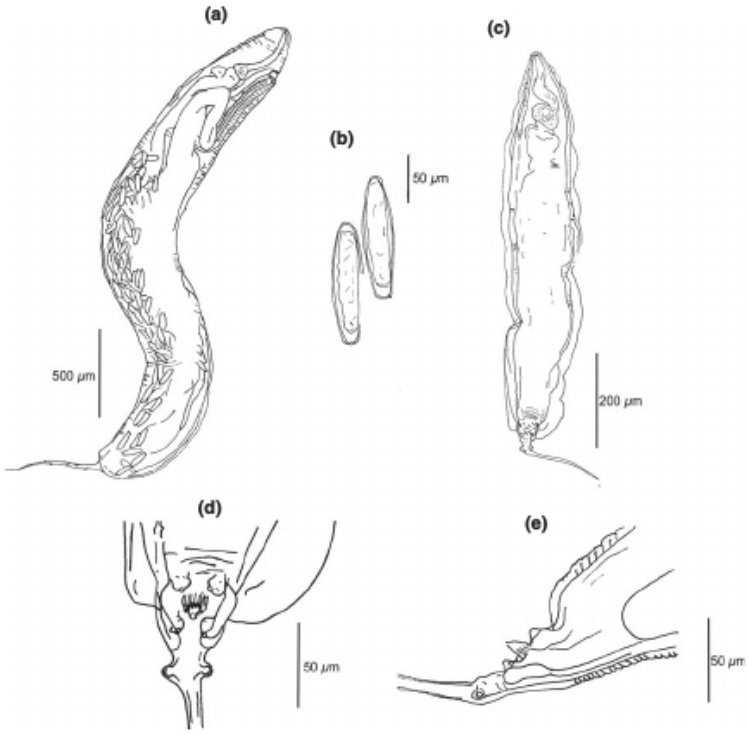

Fig.5.10- Drawings of female (a–b) and male (c–e) *Spauligodon atlanticus*. a, General view of the female; b, eggs; c, general view of the male; d, ventral view of the caudal extremity; e, lateral view of the caudal extremity.

Re-description

Small-sized nematodes with cylindrical body with tapering anterior extremities and ending with a posterior filiform tail. Sexually dimorphic, with males approximately one-third the size of gravid females. Cuticle with distinct transversal striations more marked in males, starting after the lips until the posterior extremity. Single lateral alae in males and double lateral alae in females. Mouth opening triangular, enclosed by six labial plates in males and three slightly bilobed lips in females.

Male: Small, filiform nematodes. Cuticle with distinct transversal striations, starting after the circumoral ring until the posterior extremity. Mouth opening triangular, enclosed by six equal overlapping labial plates and surrounded by a circumoral ring with two amphids located in opposite sites. Excretory pore at the end of the first third of the body, surrounded by a robust cuticular ring. Very narrow lateral alae at its start, but progressively extending along the body, reaching its maximum width with auricular shape, projected on both sides of the cloaca. Three pairs of small mammiliform caudal papillae, first two enclosed by caudal alae, third pair situated at base of tail directed outward, not enclosed by caudal alae. First pair, pre-anal, lies in higher area of posterior end, consisting of two small spherical papillae, postero-laterally directed. Second pair, postanal, similar to first pair, but slightly larger. Third pair consisting of two larger prominent and pedunculated papillae. Genital cone situated in the mid-ventral line, with an enlarged cuticularized proconus with double papillae and with two lateral side pieces associated with the proconus,

surrounded by a pleated membranous curtain with coiled edges. Posterior end extending into an aspinose, filiform tail. Spicule absent.

Female: Filiform nematodes, twice the size of conspecific males. Oral opening surrounded by three slightly bilobed lips with two amphids and no visible labial papillae. Cuticle with transversal striations, starting after the lips until the posterior extremity. Very thin, but well-defined double lateral alae extending along the body. Excretory pore and vulva opening at prebulbar level, surrounded by robust cuticular rings. Long, thick, muscular vagina, directed posteriorly. Opistodelphic females, with ovaries located behind the vulva. In fully gravid females, uterus extends anteriorly slightly past the vulva and posteriorly almost reaching the end of the body. Tail filiform, with six to nine cuticular spines. Asymmetrical eggs, with truncated extremities and with polar cap in each pole.

Distribution

Lanzarote and Fuerteventura from Canary Islands, Spain.

Host species

This species has been identified from the intestine of the lizards *Gallotia atlantica atlantica* (Peters and Doria, 1882), *G. a. laurae* Castroviejo et al., 1985 and *G. a. mahoratae* Bischoff, 1985.

Genetic and phylogeographic remarks

Spauligodon atlanticus is a monophyletic clade (Fig.5.2), not directly related to *Spauligodon occidentalis* sp. nov. (Jorge et al. 2011). *S. atlanticus* is phylogenetically more closely related to *Spauligodon* sp. from the southern part of the Iberian Peninsula and from Morocco, both parasitizing lizards of the genus *Podarcis* Wagler, 1830 (Jorge et al. 2011). This species is present in the eastern, older islands of the Canarian archipelago (see Distribution). GenBank accession numbers: JF829230, JF829232 (18S rRNA), JF829249–JF829251 (28S rRNA), and JF829272–JF829285 (COI).

Discussion

Previous phylogenetic analyses showed that what was described as *Spauligodon atlanticus*, actually consisted of two genetically different, unrelated species (Jorge et al. 2011) with the overall morphological similarity between the specimens analysed suggesting cryptic species. In the original description by Astasio-Arbiza et al. (1987), only specimens found in *G. a. atlantica* from Lanzarote, in the eastern Canary Islands, were analysed. Later studies identified *Spauligodon* specimens present in the gut of other species of *Gallotia* as *S. atlanticus sensu lato* (Martin and

Roca 2005; Jorge et al. 2011), with no attempt to analyse possible intraspecific morphological variation between the original description and the specimens found in the new host species. The overall similarity between *S. occidentalis* sp. nov. and *S. atlanticus sensu stricto* can be observed both in females and in males. For example, in both species, females have the vagina opening past the excretory pore at prebulbar level, filiform tails with cuticular spines and asymmetrical eggs with truncated extremities, while males have no spicule and show aspinose tails, lateral alae with auricular shape at the posterior end, spherical genital papillae and a genital cone with an enlarged cuticularized proconus with two side pieces, surrounded by a pleated membranous curtain. However, the combination of morphometrics and SEM allowed us to detect phenotypic differences between the two lineages. For this differentiation, the majority of the linear measurements were shown to be important. *Spauligodon occidentalis* sp. nov. is generally larger than *S. atlanticus*, in both males and females and with several morphological features presenting a more posterior position. Inclusion of measurements of the caudal extremity was also found to be important in the morphometric analysis and should be added to the features traditionally measured for these nematodes. According to our results, *Spauligodon occidentalis* sp. nov. males have a comparatively larger caudal extremity end (CT1 and CT2) and a larger third pair of caudal papillae, when compared to *S. atlanticus sensu stricto* (Fig.5.11). Regarding females, they also presented a comparatively smaller tail and more discrete double lateral alae than the *S. atlanticus sensu stricto* females. Local morphological differentiation was also detected for both males and females within each species, but this was less pronounced than the differentiation detected between the two species. Although several mechanisms including drift or isolation might account for such intraspecific variation, current evidence does not allow reaching further conclusions.

The combination of different methodologies allowed us to distinguish between what was first identified as probable cryptic species. However, the overall similarity between these two species is notable, particularly given their genetic distinctiveness. Although *S. atlanticus sensu stricto* appears as more closely related to *Spauligodon* sp. from the southern part of the Iberian Peninsula and from Morocco (parasites of *Podarcis hispanica sensu stricto* and *P. vaucheri*, respectively; Jorge et al. 2011), the lateral alae are wider at the posterior end with an auricular shape in *S. atlanticus*, with the second pair of caudal papillae having a different shape and differing also from *Spauligodon* sp. of *P. hispanica sensu stricto* by a smaller but wider genital cone. However, these later nematodes may represent an undescribed species, which still lacks a detailed morphological study and eventually a formal description. In the case of *Spauligodon occidentalis* sp. nov., it differs from *S. lacertae* in the presence of tails with cuticular spines in females, which are not present in *S. lacertae* females, and in the different shape of the second pair of caudal papillae and lateral alae with a narrower width in males. However, given the considerable geographical distance between them, they are probably not the closest species for comparison. It must be stated that to determine

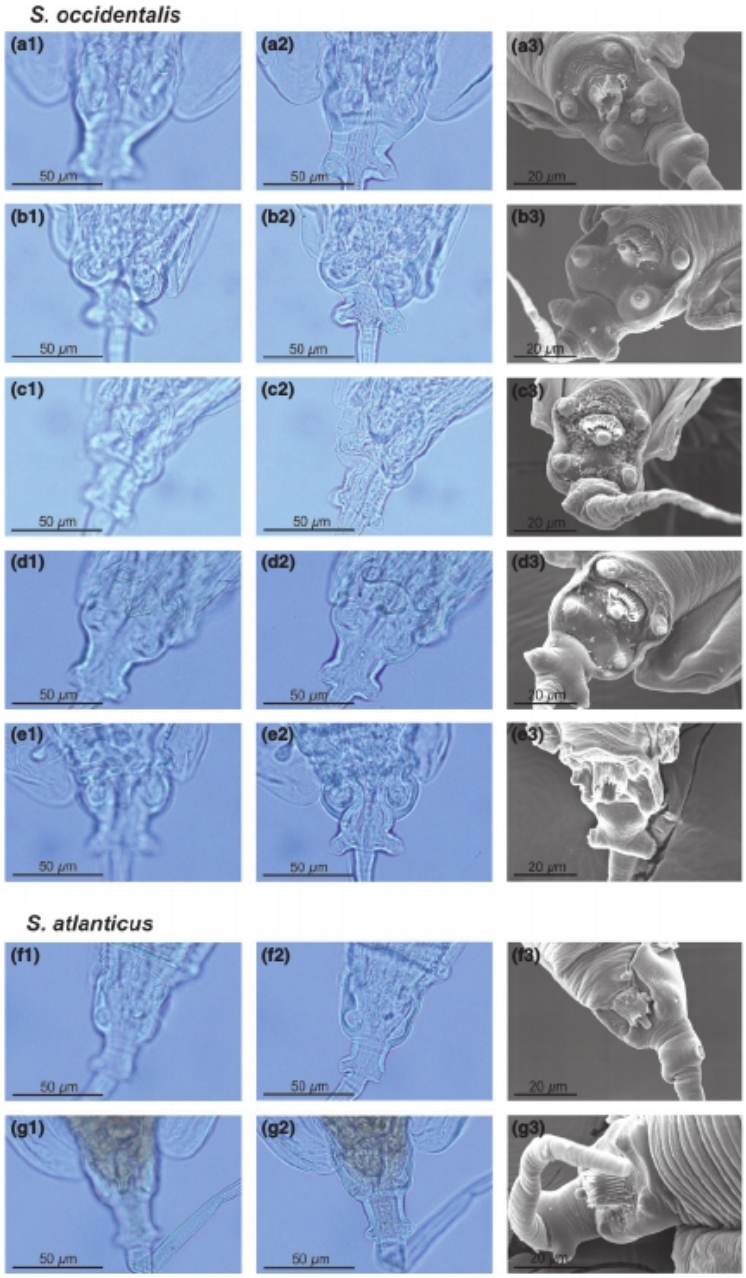

Fig.5.11- Light microscope micrographs (1–2) of the ventral view of the caudal extremity and their respective scanning electron micrograph (3) from *Spauligodon* males from all populations analysed from the western and eastern lineages. a, El Hierro east; b, El Hierro west; c, La Gomera; d, La Palma; e, Tenerife; f, Lanzarote; g, Fuerteventura; 1, focus on genital cone and first and second pair of caudal papillae; 2, focus on the third pair of caudal papillae. See Fig.5. 1 for more details on the localities.

morphological evolutionary patterns (ancestral versus derived character states) in these nematodes, their phylogeny needs first to be resolved, which will require the identification of all closely related species. The morphological resemblance between *S. occidentalis* sp. nov. and *S. atlanticu*s could be the result of morphological stasis or evolutionary convergence. This issue only could be fully accessed by analysing the morphology of a wide data set of species placed in a more complete phylogeny of the genus, which is out of the scope of this study. Nevertheless, given that both species infecting *Gallotia* lizards are not sister taxa (Jorge et al. 2011 and Fig.5.2) and

that the genetically closest species (*S. lacertae* and *Spauligodon* sp., Jorge et al. 2011) are morphologically different, we here favour evolutionary convergence as the most parsimonious scenario. The host genus *Gallotia* arrived to the Canary islands between 17 to 20 Myr (Cox et al. 2010) and presents a number of unique characteristics (e.g. large body size, karyological 2n = 40 chromosomes, strong trend to herbivory) that separate them from other members of the family Lacertidae (Arnold 1989; Arnold et al. 2007). Poulin (2011b) argued that parasite evolution has often been shaped by convergence. Convergent morphologies among divergent parasite species may be expected, due to adaptations to functionally similar internal or external environments of many host species (Perkins et al. 2011). The characteristics of the host species, the *Gallotia* lizards, could be the common factor responsible for the morphological similarity between *S. atlanticus* and *S. occidentalis* sp. nov.. In this respect, convergence is indeed a critical issue in systematics, since it can potentially mislead phylogenetic reconstruction methods based on morphological characters, for example, by causing the analyses to group distantly related organisms that share similar habitats (Wiens et al. 2003). On the other hand, traditional parasite descriptions often rely only on specimens found in a single host species (or even a single host specimen) from one locality neglecting intraspecific variation. Several *Spauligodon* species have been described in recent years (e.g. Bursey and Goldberg 2011, 2012) based exclusively on morphological characters, but cannot be easily placed in a phylogenetic framework. Furthermore, descriptive parasitological studies that only rely on morphology to identify species may underestimate the true diversity, which can be uncovered with a molecular approach. Nevertheless, we must remain cautious about how easy it is to detect new species by means of molecular tools. Incorporation of all relevant information into species descriptions will not only strengthen parasite systematics, but also contribute towards a better knowledge of host-parasite interactions.

Acknowledgements

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Supporting Information

Additional Supporting Information can be found in the Appendix D.

Data D.1. Descriptive statistics for all the linear measurements of adult specimens from the different localities included in this study.

CHAPTER 6

Where to look? Integrating molecular and morphological approaches to select the best morphological tool-box to study *Spauligodon* (Nematoda: Pharyngodonidae) diversity.

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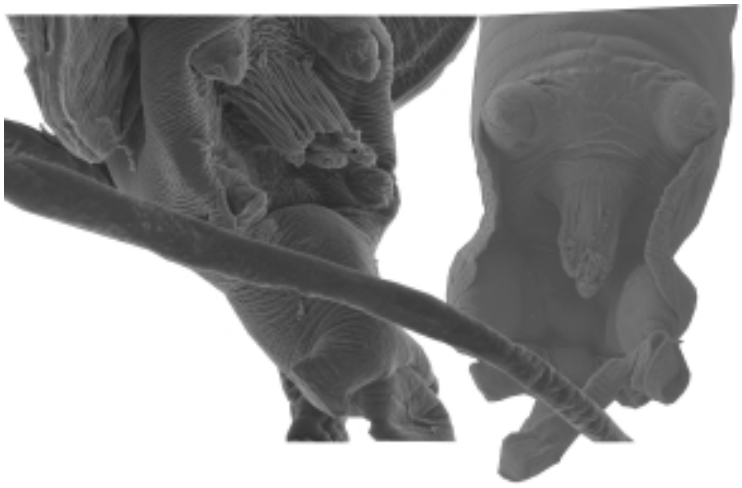

Spauligodon spp. male posterior extremities

Abstract

Understanding the diversity of living organisms involves species identification and the assessment of their phylogenetic relatedness. These two objectives are tangled and part of a common aim. Only by combining all sources of evidence can one better assess biological diversity. Parasites are expected to present reduced morphological diversity and be prone to convergent evolution. In this study, we combined molecular and morphological data to discriminate between the lineages of the parasitic nematode *Spauligodon*. Phylogenetic relationships among *Spauligodon* taxa were inferred using Bayesian methods. Morphological diversity within this taxon was first assessed by means of scanning electron microscopy. A morphological dataset of phylogenetically assessed specimens was then assembled for several male characters, and a discriminative analysis was performed. A phylogenetic comparative method was used to assess how morphological characters reflected the relatedness between lineages, and how they have evolved. Although morphological traits did vary between lineages, predictive power in lineage assignment was low. Three of the selected morphological characters revealed a strong phylogenetic signal. For all traits, morphological disparity seems to have accumulated linearly through time. Assessing how morphological traits evolved facilitates the selection of informative characters, decreasing sources of incongruence in integrative approaches.

Introduction

"*What is it?*" is one of the main questions contemplated in the field of parasitology systematics, laying its foundation (Littlewood 2011). Earlier approaches to parasite species identification mainly relied on morphological traits. Therefore, the ability to identify parasites and discriminate between species was, and remains, dependent on the knowledge and experience of parasite taxonomists. Since the implementation of molecular tools, parasite identification has gained a new perspective, and as a consequence, there has been an increase in the number of species recognised. However, many of the studies that suggest cryptic speciation are not followed by proper morphological studies, which are needed in order to provide an appropriate and formal description of the newly discovered species, contributing to the so-called "taxonomic crisis" (Dayrat 2005). This increase in taxonomic uncertainty is counterproductive to progressing research and synthesis in parasite systematics, while the problem goes far beyond the field of parasitology (Dayrat 2005; Agnarsson and Kuntner 2007; Padial et al. 2010). No single source of information should serve to describe diversity since organisms are neither only molecules nor just their morphology. Embracing one source of information to the detriment of the other does not serve the common aim of describing and understanding biodiversity. Both morphological and molecular approaches have their own virtues and pitfalls (Perkins et al. 2011), and actually other sources of information may also benefit taxonomy (Fonseca et al. 2008). An integrative and evolutionary framework provides taxonomists

with more tools to fulfil their main goal (Padial et al. 2010; Razo-Mendivil et al. 2013). New methods have been developed in the last few years to automate species delimitation, providing new insights in determining independent evolutionary lineages (Fujita et al. 2012; Miralles and Vences 2013; Solís-Lemus et al. 2014). Nevertheless, integrating several sources of evidence, i.e. molecular and morphological data for species delimitation, while necessary, is not straightforward, and incongruence across the results may arise (Carstens et al. 2013, DeBiasse and Helberg 2015). Uninformative morphological characters (Lee 2001), as well as differential rates of change (Dávalos et al. 2012), can contribute to such incongruence.

One may argue that parasites often present a simplified morphology due to their lifestyle, but this view is very limited, and morphologically distinct morphotypes of parasites with no observed genetic variation, have been identified [Blasco-Costa et al. 2010; Jorge et al. 2014 (Chapter 4)]. On the other hand, parasites species may look very similar, and in fact, morphological cryptic species are common (Nadler and Pérez-Ponce de León 2011; Poulin 2011). However, distinguishing between species that are morphologically similar may be constrained by our (in)ability to identify reliable diagnostic morphological characteristics. New high-resolution tools are available and when integrated with a detailed morphological analyses, under-appreciated morphological diversity can be uncovered [De Ley et al. 2005; Jorge et al. 2013 (Chapter 5)]. But not all morphological traits may be suitable for species diagnosis. Diagnostic morphological traits should reflect the organisms long-term evolutionary history, undergoing divergent evolution. In this case, the set of morphological traits are expected to have a strong phylogenetic signal. However, phylogenetic signals are often diluted by processes that lead to homoplasy, such as reversals, parallel evolution and convergence (Klingenberg and Gidaszewski 2010). How a morphological trait is shaped and/or restrained by evolutionary history reflects in its suitability for species discrimination.

Nematodes are highly diverse, complex and specialised, despite the simple underlying anatomy (De Ley 2006). Nevertheless, morphological species recognition is controversial (Fonseca et al. 2008). This study focuses within a taxon of parasitic nematodes, genus *Spauligodon*. The *Spauligodon* parasitic taxon has been the focus of several evolutionary studies, and along the way several new undescribed lineages have been identified (Falk and Perkins 2013; Chapter 2; Chapter 3). *Spauligodon* species determination is based on the structure of the male caudal extremity, since females seem to be very similar between groups. The caudal extremity of the male of the Pharyngodonidae also seems to better show the phylogenetic relationships of the group, which may be because it is related with reproduction (Anderson et al. 2009). While recent studies have tried to identify new diagnostic morphological characters [Jorge et al. 2013 (Chapter 5)], it is still unclear in which manner these characters evolved within this parasitic nematode taxon.

In this study, we used a combined approach to investigate the reliability of a set of adult male

morphological traits as a diagnostic tool for lineage discrimination of the genus *Spauligodon*. We estimated phylogenetic relationships within this parasitic taxon with Bayesian methods, and delimited new lineages. Moreover, scanning electron microscopy was used as an exploratory tool to identify new morphological characters, and as a result, several morphological traits were quantified in adult *Spauligodon* males. We statistically assessed the ability to predict group membership with the selected morphological traits. Phylogenetic comparative methods were then used to determine the tempo and mode of evolution of those traits and how useful they were for the taxonomy of this parasitic nematode taxon.

Material and Methods

Spauligodon lineage identification

Nematodes of the genus *Spauligodon* were collected from faecal pellets, which were obtained from 12 reptile host species, collected from 15 localities (Table 6.1). Samples were collected and processed as described in Jorge et al. [2014 (Chapter 4)]. Individual nematode specimens from each locality and from each different host were genetically analysed. Extraction of genomic DNA was performed on individual nematodes using the PureLink® Genomic DNA Kit (Invitrogen, Invitrogen New Zealand Ltd, Auckland, New Zealand). Two different DNA fragments were analysed: the nuclear 28S ribosomal RNA (28S) and the mitochondrial cytochrome oxidase subunit I (COI). DNA amplification and sequencing were performed as described in Jorge et al. [2014 (Chapter 4)]. Nematode sequences were edited and trimmed in GENEIOUS v8.1.4 (http://www.geneious.com; Kearse et al. 2012). Additional *Spauligodon* DNA sequence data published in previous studies was also included in the molecular datasets (28S: 49 sequences, COI: 58 sequences; Table 6.1). For both datasets the nematodes *Parapharyngodon echinatus* and *Thelandros tinerfensis* were used as outgroups. Sequences were aligned in GENEIOUS v8.1.4 (http://www.geneious.com; Kearse et al. 2012) with MAFFT (Katoh et al. 2002) selecting the E-INS-i algorithm, using the default parameters (scoring matrix = 200 PAM/k = 2; gap open penalty = 1.53) for the 28S dataset, and the G-INS-i algorithm using the default parameters (scoring matrix = 200 PAM/k = 2) for COI dataset. To determine the correct reading frame, the COI alignment was translated to amino acids, specifying invmtDNA gene code, in GENEIOUS v8.1.4 (http://www.geneious.com; Kearse et al. 2012). Nine samples were lacking sequence data for the 28S. Because missing data are expected to have minor impact on the accuracy of phylogenetic analysis (Wiens and Morril 2011), those sequences were included in the concatenated COI and 28S dataset coded as “?”.

Phylogenetic relationships among *Spauligodon* putative taxa were inferred with a Bayesian inference method in MRBAYES v3.2.6 (Ronquist et al. 2012), as implemented in the CIPRES

Table 6.1 Nematode specimens included in the phylogenetic analysis, including their respective host species and locality of origin, and GenBank accession number.

Code	Lineage	Host	Locality	Genbank		Reference
				28S	COI	
KF029393	*S. anolis*	*Anolis* sp.	Puerto Rico	-	KF029393	Falk and Perkins 2013
S16536MI	*S. atlanticus*	*Gallotia atlantica*	Fuerteventura Is., Spain	KJ778096	KJ778108	Jorge et al. 2014
S1492F	*S. atlanticus*	*Gallotia atlantica*	Lanzarote, Spain	JF829250	JF829274	Jorge et al. 2011
S11212F	*S. atlanticus* Type Mor	*Podarcis hispanica*	Algeria	x	x	Chapter 3
S1757MA	*S. atlanticus* Type Mor	*Podarcis vaucheri*	Morocco	x	x	Chapter 2
S11031F	*S. atlanticus* Type Mor	*Podarcis vaucheri*	Morocco	x	x	Chapter 2
S14717MA	*S. auziensis* Type1	*Tarentola mauritanica*	*Morocco*	x	x	Chapter 2
S21269F	*S. auziensis* Type1	*Tarentola mauritanica*	Portugal	x	x	Chapter 3
S14720MA	*S. auziensis* Type2	*Tarentola mauritanica*	Morocco	-	x	This study
SAP1F	*S. cabrerae*	*Podarcis lilfordi*	Menorca Is., Spain	x	x	Chapter 2
SM1MA	*S. cabrerae*	*Podarcis lilfordi*	Menorca Is., Spain	-	x	This study
S12023F	*S. cabrerae*	*Podarcis pityusensis*	Ibiza Is., Spain	x	x	This study
S24217MI	*S. cabrerae*	*Tarentola desertii*	Morocco	x	x	This study
S7930MA	*S. carbonelli*	*Algyroides marchi*	Spain	x	x	Chapter 3
S12492MA	*S. carbonelli*	*Iberolacerta horvathi*	Slovenia	x	x	This study
S32Ic	*S. carbonelli*	*Podarcis bocagei*	Portugal	x	x	This study
S71	*S. carbonelli*	*Podarcis bocagei*	Portugal	x	x	This study
S16147MA	*S. carbonelli*	*Podarcis erhardii*	Serbia	x	x	This study
S13432MA	*S. carbonelli*	*Podarcis hispanica* PH2	Portugal	KJ778092	KJ778111	Jorge et al. 2014
S20400MA	*S. extenuatus*	*Timon lepidus*	Portugal	x	x	Chapter 2
S20403F	*S. extenuatus*	*Timon lepidus*	Portugal	x	x	This study
SLm28F	*S. lacertae*	*Lacerta media*	Armenia	JF829255	JF829287	Jorge et al. 2011
S10057F	*S. lacertae*	*Lacerta strigata*	Armenia	JF829252	JF829286	Jorge et al. 2011
S2597F	*S. nicolauensis*	*Tarentola bocagei*	São Nicolau Is., Cape Verde	JF829243	JF829265	Jorge et al. 2011
S2828F	*S. nicolauensis*	*Tarentola nicolauensis*	São Nicolau, Cape Verde	JN619358	JN619359	Jorge et al. 2012
S22475F	*S. nicolauensis*	*Tarentola substituta*	São Vicente Is., Cape Verde	x	x	Chapter 3
S16492F	*S. occidentalis*	*Gallotia atlantica*	Fuerteventura, Spain	x	x	Chapter 2
S2467MA	*S. occidentalis*	*Gallotia caesaris*	El Hierro Is., Spain	JF829258	JF829303	Jorge et al. 2011
S2447MA	*S. occidentalis*	*Gallotia caesaris*	El Hierro Spain	JF829261	JF829306	Jorge et al. 2011
S19330MA	*S. occidentalis*	*Gallotia galloti*	La Palma, Spain	x	x	Chapter 2
S19253MA	*S. occidentalis*	*Galotia galloti*	Tenerife Is., Spain	x	x	Chapter 2
S7456MA2	*S. paratectipenis*	*Hemidactylus turcicus*	Ibiza Is., Spain	x	x	Chapter 3
S10420F	*S. saxicolae* Type Arm	*Darevskia nairensis*	Armenia	JF829245	JF829268	Jorge et al. 2011
S9902F	*S. saxicolae* Type Arm	*Darevskia unisexualis*	Armenia	JF829246	JF829266	Jorge et al. 2011
SFA502M	*S. saxicolae* Type Ir	*Darevskia chlorogaster*	Iran	x	x	Chapter 3
SDr3KMA	*S. saxicolae* Type TK	*Darevskia rudis*	Turkey	-	KJ778112	Jorge et al. 2014
S22991MA	S. sp. Type A	*Podarcis erhardii*	Santorini Is., Greece	x	x	This study
S7158F2	S. sp. Type A	*Podarcis sicula*	Menorca, Spain	x	x	Chapter 2
S15123MA	S. sp. Type A	*Podarcis sicula*	Sardinia Is., Italy	-	x	This study
S15437F	S. sp. Type A	*Podarcis sicula*	Sardinia, Italy	x	x	Chapter 2
S23716MA	S. sp. Type A	*Podarcis wagleriana*	Sicily Is., Italy	x	x	This study
S9168F	S. sp. Type A	*Scelarcis perspicillata*	Menorca Is., Spain	x	x	Chapter 3
SJCB6417F	S. sp. Type AB	*Tarentola ephippiata*	Mauritania	x	x	Chapter 2
S14741F	S. sp. Type AC	*Sthenodactylus sthenodactylus*	Morocco	-	x	This study
SP075F	S. sp. Type AD	*Phelsuma lineata*	Madagascar	x	x	Chapter 3
S5874MA	S. sp. Type B	*Podarcis sicula*	Portugal	-	x	This study
S19478MA	S. sp. Type CanA2	*Chalcides coeruleopunctatus*	El Hierro, Spain	x	x	Chapter 2
S23142F2	S. sp. Type CanA2	*Chalcides viridanus*	Tenerife Is., Spain	x	x	Chapter 2

x, unsubmitted sequence data to GenBank.

Table 6.1 Cont.

Code	Lineage	Host	Locality	Genbank		Reference
				28S	COI	
S19352MA	S. sp. Type CanA2	*Chlacides coeruleopunctatus*	La Gomera Is., Spain	x	x	Chapter 2
SL2H3F	S. sp. Type CanC	*Chalcides coeruleopunctatus*	El Hierro Is., Spain	x	x	Chapter 2
S23071F	S. sp. Type CanD1	*Tarentola boettgeri*	Gran Canaria Is., Spain	x	x	Chapter 2
S23168F4	S. sp. Type CanD2	*Tarentola gomerensis*	La Gomera Is., Spain	x	x	Chapter 2
S15448MA	S. sp. Type D	*Podarcis tiliguerta*	Sardinia Is., Italy	x	x	Chapter 2
S15120MA	S. sp. Type D	*Podarcis tiliguerta*	Sardinia, Italy	KJ778100	KJ778109	Jorge et al. 2014
S11323F2	S. sp. Type K	*Quedenfeldtia trachyblepharus*	Morocco	x	x	Chapter 3
S23755F	S. sp. Type O	*Acanthodactylus erythrurus*	Morocco	x	x	Chapter 3
S24080MA	S. sp. Type O	*Acanthodactylus sp.*	Morocco	x	x	Chapter 3
S9582F	S. sp. Type P	*Archaeolacerta bedriagae*	Corsica Is., France	x	x	Chapter 3
S15043MA	S. sp. Type R	*Chalcides ocellatus*	Sardinia Is., Italy	x	x	Chapter 2
S3320F	S. sp. Type R	*Chalcides sp.*	Morocco	x	x	Chapter 2
SG9M	S. sp. Type U	*Tarentola gigas*	Raso Is., Cape Verde	KJ778095	KJ778104	Jorge et al. 2014
S14167F	S. sp. Type V	*Tarentola desertii*	Morocco	x	x	Chapter 2
S14189MA	S. sp. Type V	*Tarentola desertiii*	Morocco	-	x	This study
S14714MA	S. sp. Type V	*Tarentola sp.*	Morocco	x	x	This study
S5011F	S. sp. Type J	*Atlantolacerta andreanskyi*	Morocco	x	x	Chapter 3
RP1008	*S. trimorphi*	*Oligosoma otagense*	New Zealand	x	x	Mocket et al.unpublished
S12489F	S. Type P	*Iberolacerta horvathi*	Slovenia	x	x	Chapter 3
S20448F	S. Type P	*Iberolacerta monticola*	Portugal	x	x	Chapter 3
S20571F	S. Type P	*Podarcis hispanica* PH1A	Portugal	x	x	Chapter 2
S16078F	S. Type P	*Podarcis taurica*	Serbia	x	x	Chapter 3
S14897	S. Type P	*Scelarcis perspicillata*	Morocco	x	x	Chapter 3
S14988F	S. Type P	*Scelarcis perspicillata*	Morocco	x	x	Chapter 3
SFA474F	S. Type Q	*Iranolacerta brandtti*	Iran	x	x	Chapter 3
T19408	*Thelandros tinerfensis*	*Tarentola gomerensis*	La Gomera, Spain	KJ778089[a]	X[b]	[a]Jorge et al. 2014 / [b]Chapter 2
P1328	*Parapharyngodon echinatus*	*Gallotia atlantica*	Fuerteventuraa, Spain	JF829240	JF829262	Jorge et al. 2011

x, unsubmitted sequence data to GenBank. Is, Islands.

Science Gateway v3.3 (Miller et al. 2010) from the concatenated COI and 28S dataset. Following Chapter 3, the 3rd COI codon position was excluded from the analysis due to saturation issues. A partitioned model based on genes and codon positions was implemented. For each partition, we specified a model *a priori* allowing for the estimation of base frequencies, the proportion of invariable sites and rate-variation across sites with a gamma distribution. A reversible-jump Markov chain Monte Carlo (MCMC) was used to integrate over the pool of all 203 possible reversible 4×4 nucleotide models. One hundred million MCMC generations were sampled every 1000th step and the first 25% were discarded as burn-in. We ran two independent runs each with 1 cold and 3 heated chains (T=0.04) and pooled the samples after burn-in was removed. Mixing and convergence of each run was monitored through the statistics provided in MRBAYES [values of standard deviation of partition frequencies (<0.01), potential scale reduction factors (PSRF) (1.00), effective sample sizes (ESS) (>200)] and in TRACER v1.6 (Rambaut et al. 2014). We defined lineages by visually inspecting the inferred phylogeny for well supported clades. The robustness of

the lineages were further enhanced by constricting COI genetic distances between groups. Defining a threshold of molecular divergence in nematodes is not straightforward. Nematode literature suggests that intraspecific and interspecific differences in mtDNA of nematodes ranges between 0.3% - 8.6% and 4.8 – 20% respectively, (Blouin 2002; Hu and Gasser 2006). In our study we set the threshold of mitochondrial interspecific difference for well supported clades, to above 7% (uncorrected *p*-distance). This value was based on estimated distances between described closely related *Spauligodon* species (i.e. *S. lacertae* and *S. extenuatus* 7% uncorrected *p*-distance). However, in some very structured clades, a maximum of 9% (uncorrected *p*-distance) intraspecific divergence was allowed (Fig.6.1). This within-group divergence can be a direct result of the sample size. Estimates of evolutionary divergence for the COI pairwise uncorrected

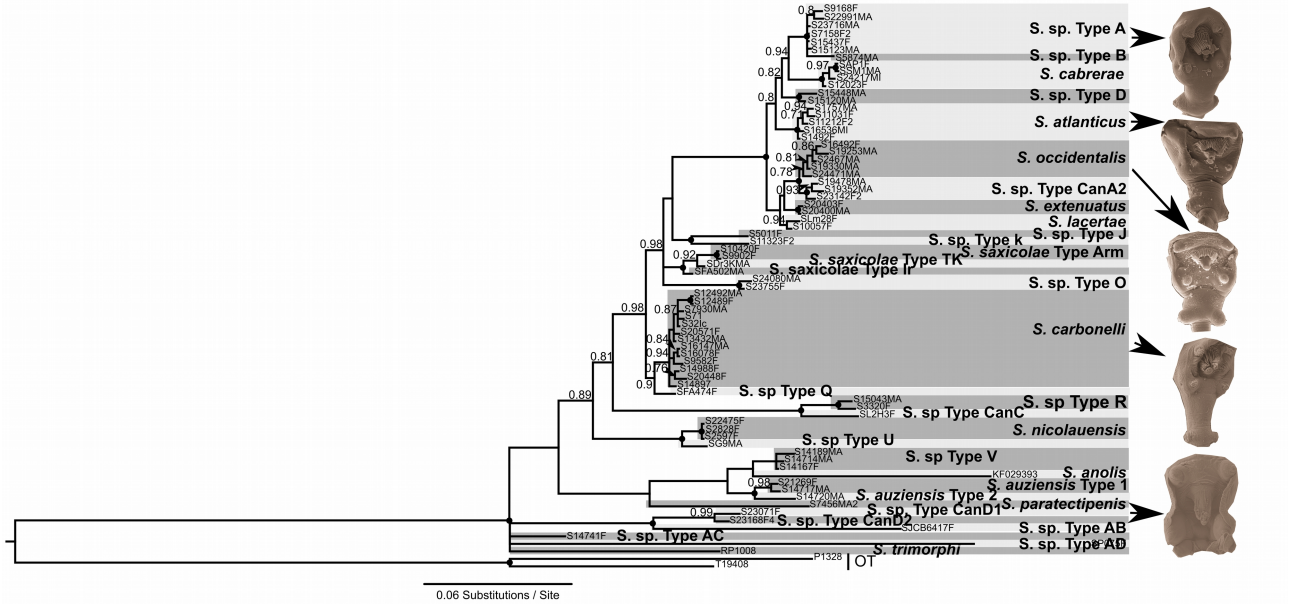

Fig.6.1- Bayesian 50% majority-rule inference tree for the concatenated 28S and COI parasite dataset as well as scanning electron micrographs of the caudal extremity of some *Spauligodon* lineages. Shaded rectangles indicate delimited *Spauligodon* lineages. Branch labels show posterior probabilities (values below 0.75 not shown). Filled circle on the nodes correspond to a posterior probability value of 1. Outgroups (OT).

differences (*p*-distance) between and within defined lineages were made in MEGA v6 (Tamura et al. 2013). A Bayesian Markov chain Monte Carlo (MCMC) coalescent framework was also performed over a restricted dataset containing only one representative per *Spauligodon* delineated lineage without outgroups. The method was implemented in BEAST v2.3.0 (Bouckaert et al. 2014) over the concatenated COI (excluding the 3rd codon position) and 28S dataset. Following Chapter 3, an uncorrelated Lognormal relaxed clock was implemented with the root arbitrarily set to a uniform distribution between 85 and 95. The site model was inferred during the MCMC, estimating all three components of the site model using reversible jump, but grouping within transitions and within transversions (bModelTest package of BEAST; Bouckaert et al. 2015). The birth–death constant speciation and extinction rates model (Nee et al. 1994, Gernhard 2008) was set as tree

prior. We specified a diffuse "uninformative" but proper priors characterising the rate of evolutionary change with a gamma distribution (Alpha = 0.001 and Beta = 1000) on the mean and with a exponential distribution (Mean = 0.333) on the standard deviation. Three independent MCMC analyses were run for 100 million generations with a sampling frequency of ten thousand. Convergence diagnostics were examined for the combined runs in TRACER v. 1.6 (Rambaut et al. 2014). Sampled trees were combined in LOGCOMBINER v2.3.0 (Bouckaert et al. 2014), discharging the first 25% of the samples in each tree file. The most probable trees were summarized into a maximum clade credibility tree using TREEANNOTATOR v2.3.0 (Bouckaert et al. 2014).

Morphological data

Adult female (15) and male (33) nematodes from the same localities, and when possible, from the same host as the parasites that underwent genetic assessment, were selected for scanning electron microscopy analysis. This analysis has previously proven to be a good preliminary step to identify possible diagnostic morphological characters in this nematode group [Jorge et al. 2013 (Chapter 5)]. The specimens included in this analysis represented 12 of the defined *Spauligodon* lineages (see Results), which were distributed across the inferred phylogeny. Nematode specimens were dehydrated through ethanol series, and then dried to a critical point. Specimens were coated with AuPd and examined with a Zeiss Ultra Plus Field Emission scanning electron microscope coupled with Zeiss SmartSEM image navigation. Preliminary examination of scanning electron micrographs led to the inclusion of six new morphological characters to the morphometric analyses. Adult male specimens from several of the delimited *Spauligodon* lineages were mounted and photographed as described in Jorge et al. [2013 (Chapter 5)]. In total, 100 specimens, belonging to 24 *Spauligodon* lineages, were photographed and were measured in ImageJ (Schneider et al. 2012). These specimens were obtained from the current study and from previous studies [Jorge et al. 2011; 2012; 2013 (Chapter 5); 2014 (Chapter 4)]. We used the linear measurements in the posterior extremity of male specimens, which are described in Jorge et al. [2013 (Chapter 5); Fig.6.2]: caudal trunk width at its widest point (CT1) and narrowest point (CT2), insertion of the third pair of caudal papillae, width of one of the papilla of the third pair of caudal papillae at the tip (3p1), middle (3p2), insertion point (3p3) and the length (3pl). In addition, six new traits were also measured here: posterior extremity length (PextL) as the measure from the 1st pair of caudal papillae until the 3rd pair of caudal papillae, the width at the level of the 2nd pair of caudal papillae (PextW1), width of the 3rd pair of caudal papillae (3pW), width of the 1st pair of caudal papillae (1pW) and width (2pW) and length (2pL) of 2nd pair of caudal papillae (Fig.6.2). However, CT1, 3p2 and 3p3 traits had to be later removed from the final morphological dataset due to numerous missing data. Body length (BL), length of oesophageal bulb (OBL) and width (OBW) were also measured as described by Jorge et al. [2013, (Chapter 5)]. The final morphological

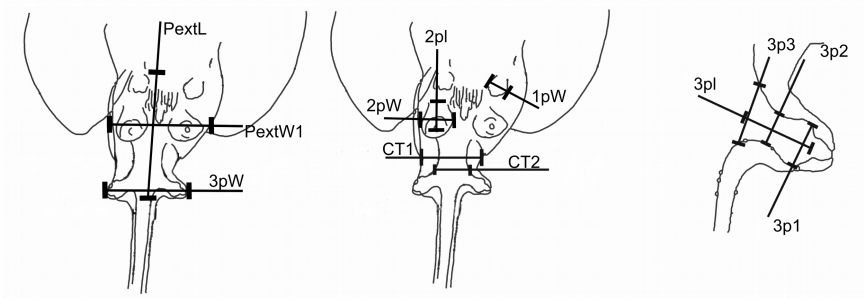

Fig.6.2- Linear measurements of male posterior extremities, which were recorded for morphological analyses with their respective designation. See material and methods for the abbreviations of morphological traits.

dataset consisted of 12 traits. [Author note: Morphological data from this study (i.e. voucher pictures) will be made available in MorphoBank in the published version of the chapter.]

Statistical analyses

All linear measurements were log-transformed prior to statistical analyses to better approximate a normal distribution and for all variables be on a similar scale. Correlations between traits were assessed with a nonparametric Spearman correlation (function rcorr, R package *Hmisc,* Harrell 2013). We used a MANOVA with a Pillai test to determine if our categorical predictor, lineage, has an effect on the multivariate morphology data (manova function of *R* package, R Core Team 2015) and also to calculate the partial eta-squared (etasq function from the *heplots* package, Fox et al. 2015). To determine if we could use the selected morphological traits to predict group membership, we performed a Linear Discriminant analysis (LDA) with a jackknife prediction. Prior to LDA analysis we assessed if each lineage had the same within-group covariance by permutation-based approach (permutest from the *vegan* package, Oksanen et al. 2012). LDA was run with the function lda from the *MASS* package (Venables and Ripley 2002). No prior probabilities were specified but instead proportional prior probabilities were assumed. The following statistical analyses were performed over the lineage mean or each trait measurement. Since size (BL) was found to be strongly correlated with several of the other morphological traits, we performed a linear regression of species mean trait measurements on species BL. The regression residuals against BL were used as size corrected measures of each of those trait values. For comparative analyses accounting for species phylogenetic relationships, we used the maximum clade credibility tree obtained in BEAST analyses. The tree was pruned to include only the 24 *Spauligodon* lineages measured in this study. To determine if the selected morphological traits variation exhibit a phylogenetic structure, we tested for phylogenetic signal for each trait separately. This analysis is important to assess if the selected morphological characters are evolving dependently from the phylogeny and therefore, indicating its suitability for taxonomic procedures. We used two different indexes: Pagel's λ (Pagel 1999) and Blomberg's *K* (Blomberg et al. 2003). Pagel's λ measures the dependence of the observed trait assuming a pure Brownian

model of evolution (BM) and it can adopt values larger than one (traits of related species are more similar than expected under the BM) (Pagel 1999; Münkemüller et al. 2012). Blombegr's *K* tests whether the observed distribution of traits exhibits more or less divergence than expected for traits evolving under Brownian motion (Blomberg et al. 2003). Values of $K \geq 1$ are indicative of phylogenetic signal equal to or greater than the expectation under the BM, whereas values of $K < 1$ indicate little or no phylogenetic signal. These two tests capture different aspects of phylogenetic signal and can sometimes lead to contrasting results with Blomberg's *K* being more sensitive to detecting subtle changes in phylogenetic signal (Münkemüller et al. 2012). Pagel's λ and Blomberg's *K* were estimated with the function phylosignal using the *picante* package (Kembel et al. 2010) with 999 randomizations. To determine how the morphological traits were evolving we fitted three evolutionary models to each trait separately. The BM (Felsenstein 1973) assumes the correlations structure among trait values is proportional to the extent of shared ancestry for pairs of species. Under this model, traits are assumed to evolve under neutral drift of character change with no selection. The Ornstein-Uhlenbeck model (OU, Butler and King 2004) fits a random walk with a central tendency that has an attraction strength proportional to the parameter alpha. In the OU model, traits evolve under a persistent stabilizing selection around an optimal trait value (1 = stabilizing selection; 2+ = divergent selection). The Early-burst model [EB, Harmon et al. 2010; also called the ACDC model (accelerating-decelerating; Blomberg et al. 2003)] fits a model where the rate of evolution increases or decreases exponentially through time. Under this model, a trait shows a pattern of rapid morphological evolution followed by relative stasis. The three evolutionary models were fit using the fitContinuous function from the *geiger* package (Harmon et al. 2008). Because measurement error can bias our inference when fitting these models towards OU, we calculated the average standard deviation within each lineage for each trait and added it as an argument of the function. To compare the three models under consideration, we converted the AICc values to Akaike weights. The model with the highest value was considered the best model (Burnham and Anderson 2002). All analyses were implemented using the package R v3.2.2 (R Core Team 2015).

Results

Spauligodon lineage identification

Spauligodon phylogenetic relationships were estimated from 71 specimens that were found within 49 different host species from different localities. For the Bayesian inference (BI) analyses, each separate run converged to an average deviation of split frequencies below 0.004. Thirty-two different lineages were defined. Genetic distances within those lineages ranged from 0% – 9% (uncorrected *p*-distance), and between lineages ranged from 7.1% to 25.3% (uncorrected *p*-distance). The BEAST Bayesian MCMC inference runs converged efficiently on a posterior mean

value for all parameters. The maximum clade credibility tree recovers a different topology for some internal branches but with low support values (posterior probability < 0.55). The positions of two branches differed between the BI tree and the maximum credibility tree. In the former these branches were basal while they were not in the later analysis. (Fig.6.1 and Fig.6.3).

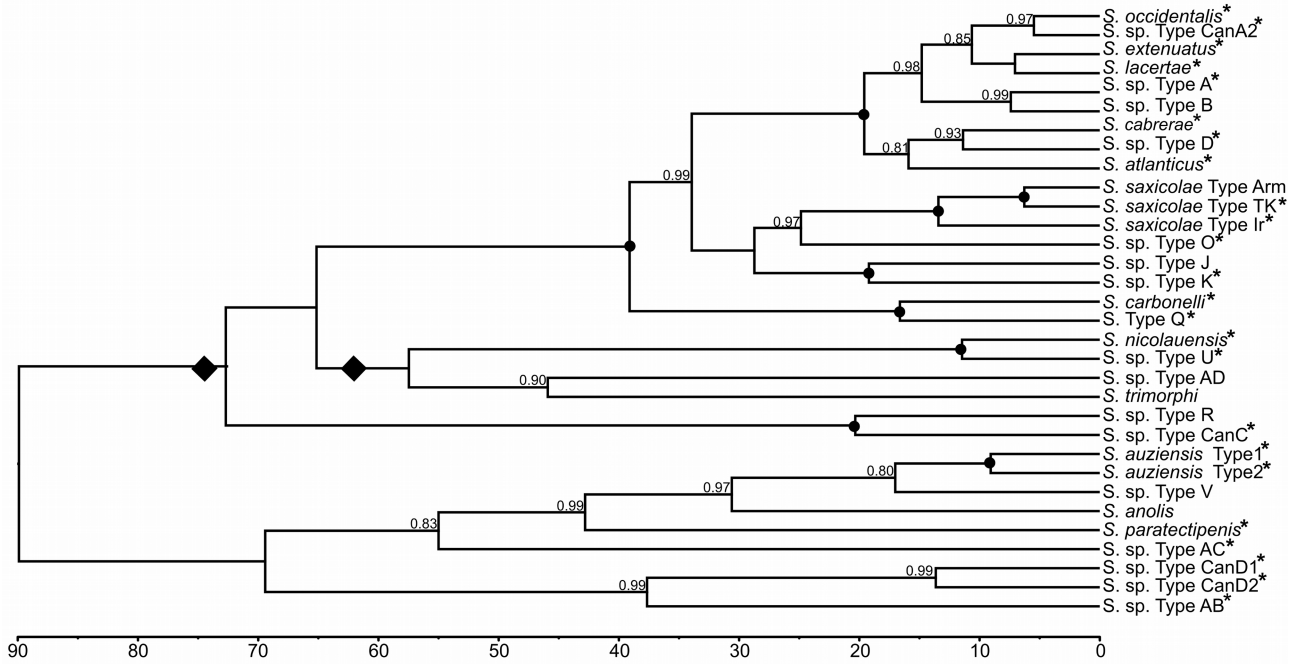

Fig.6.3- Maximum clade credibility ultrametric timescaled tree for the concatenated COI and 28S parasite dataset. Values on the axis represent relative time estimates in Mya. Branch labels show posterior probabilities (values below 0.75 are not shown). Filled circle on the node corresponds to a posterior probability value of 1. Black diamonds represent incongruent branch topologies relative to Bayesian 50% majority-rule. * *Spauligodon* lineages represented in the morphological dataset.

Statistical analyses

Descriptive statistics of morphological characters for all specimens are given in the supporting information (Data E.1, Supporting Information). BL was correlated with several of the morphological traits, with the exception of CT2 (R = 0.12, P = 0.579), EBL (R = -0.05, P = 0.802) and EBW (R = 0.02, P = 0.939). There was strong evidence that the measurements of morphological traits vary between *Spauligodon* lineages ($F_{(276,\ 912)}$ = 3.159, P < 0.0001, Pillai = 5.865, partial η^2 = 0.489). These results were also consistent when analysed for each trait separately (all cases P < 0.001). Each lineage had a similar within-group covariance ($F_{(23,\ 76)}$ = 1.543, P = 0.105). The first linear discriminant explained 42% of the between group variance in the *Spauligodon* morphological dataset. Within this trace, the characters CT2, PextL and 3pW were the ones contributing the most to group separation. The accuracy of prediction was of 59%. Most traits showed phylogenetic structure, with CT2, PextL and 3pW having the strongest phylogenetic signal (Table 6.2). For all the morphological traits, the BM model fitted the data the best, presenting the highest AICc weight (Table 6.3).

Table 6.2 Estimated phylogenetic signal for Pagel's λ and Blomberg's *K* for each morphological trait. Significant *p*-values (*P*) are highlighted in bold

Trait	Pagel's λ	*P* (Pagel's λ)	Blomberg's *K*	*P* (Blomberg's *K*)
BL	7.84E-005	1	0.358	0.150
X1pW	0.321	0.795	0.354	0.150
X2pW	0.649	0.063	**0.451**	**0.021**
X2pl	0.130	0.672	0.298	0.346
CT2	**1.023**	**0.000**	**1.843**	**0.001**
X3p1	0.583	0.089	**0.433**	**0.043**
X3pl	**0.751**	**0.013**	**0.537**	**0.005**
PextL	**0.930**	**0.000**	**1.185**	**0.001**
PextW1	0.461	0.142	0.266	0.537
X3pW	**0.887**	**0.001**	**0.846**	**0.002**
EBL	0.000	1.000	0.219	0.833
EBW	0.000	1.000	0.238	0.692

Tale 6.3 Parameter estimates and model fitting for the phenotypic traits with high phylogenetic signal (Pagel's λ >0.70 and Blomberg's K >0.70) in *Spauligodon* nematodes. The best-fit model is highlighted in bold. AICc: Akaike information criterion for small sample sizes. AICw: Akaike weight.

Trait	K	Model	AICc	AICw	Trait	K	Model	AICc	AICw
	2	**BM**	**55.450**	**0.650**		2	**BM**	**-45.270**	**0.634**
BL	3	OU	58.078	0.175	X3pl	3	OU	-42.641	0.170
	3	EB	58.078	0.175		3	EB	-42.917	0.196
	2	**BM**	**-38.572**	**0.630**		2	**BM**	**12.104**	**0.650**
X1pW	3	OU	-36.282	0.200	PextL	3	OU	14.733	0.175
	3	EB	-35.944	0.169		3	EB	14.733	0.175
	2	**BM**	**-18.880**	**0.650**		2	**BM**	**6.433**	**0.650**
X2pW	3	OU	-16.251	0.175	PextW1	3	OU	9.062	0.175
	3	EB	-16.251	0.175		3	EB	9.062	0.175
	2	**BM**	**-16.886**	**0.650**		2	**BM**	**-13.609**	**0.650**
X2pl	3	OU	-14.257	0.175	X3pW	3	OU	-10.980	0.175
	3	EB	-14.257	0.175		3	EB	-10.980	0.175
	2	**BM**	**-27.820**	**0.580**		2	**BM**	**8.613**	**0.650**
CT2	3	OU	-25.191	0.156	EBL	3	OU	11.242	0.175
	3	EB	-26.246	0.264		3	EB	11.242	0.175
	2	**BM**	**-52.978**	**0.614**		2	**BM**	**16.485**	**0.650**
X3p1	3	OU	-50.928	0.220	EBW	3	OU	19.113	0.175
	3	EB	-50.350	0.165		3	EB	19.113	0.175

Discussion

In the quest to properly describe biodiversity, integrative frameworks may be the best

conceptual and most suitable methodological tools to face this challenge (Padial et al. 2010). Molecular techniques are indeed very appealing due to their relatively fast processing time. On the contrary, morphology-based ones can be very time consuming, especially when it comes to specimens that require imaging technology. Additionally, selection of phylogenetic informative morphological characters should be conducted prior to integrative approaches of species delimitation in order to reduce possible incongruence between data sets. In this study, we followed such an approach with the parasitic nematode taxon, genus *Spauligodon.* We first inferred the phylogeny to identify *Spauligodon* nematode lineages. However, lineage discrimination within nematodes is not straightforward (Fonseca et al. 2008; Nadler and Pérez-Ponce de León 2011). Genetic variation within groups makes it difficult to determine the boundaries between intraspecific diversity and interspecific diversity. In this study, the variability in sample size within lineages also made it difficult to determine species boundaries (Fig.6.1). We choose a more conservative approach, which allowed some groups to contain high intraspecific diversity (i.e. *Spauligodon* Sp. Type CanA2 uncorrected *p*-distance = 9%). This conservative approach did not influence the morphological analysis since the lineages did not significantly differ in traits covariance measurements. Nevertheless, this conservative lineages approach allowed a clearer between groups delimitation.

The morphology of the posterior extremity of *Spauligodon* species is variable in size but especially in the shape and size of the genital papillae (Fig.6.1). However, only qualitative descriptions of this extremity are usually given [but see Jorge et al. 2013 (Chapter 5)]. In our comparative study across several *Spauligodon* lineages, we mainly focussed on quantitative morphometrics in this body region of the nematodes. While there was strong evidence that the selected measurements of morphological traits varied between *Spauligodon* lineages, their use in lineages assignment had low predictive power. Three of the morphological characters investigated, CT2, PextL and 3pW, revealed a strong phylogenetic signal. Such structure in morphological characters brings further support for their use in species discrimination, since their divergence follows phylogenetic divergence between lineages. The low prediction power for the *Spauligodon* lineage assignment can be a consequence of the other morphological traits that do not show such strong phylogenetic signal. This means that even if morphological traits do show evidence of disparity between lineages, other processes may influence their evolution. When evaluating the models that better described their divergence along the inferred phylogeny for all morphological traits, results suggested a BM model of evolution. This implies that morphological disparity accumulated approximately linearly through time, without any evidence for selection. The posterior extremity of male nematodes is associated with reproduction, possessing the reproductive structures. Genital papillae have sensory functions, but it is not clear what role the other measured characters have in sexual reproduction, even though they are an integrated part of the posterior

end of male body. However, these characters seem to follow a common evolutionary trajectory, matching a neutral drift of character change with no selection, without evidence of any particular evolutionary pressure, i.e. sexual selection.

Determining the best molecular markers to use for species discrimination within parasitic nematodes, and within parasites in general, is still highly dependent on the available markers (i.e. molecular markers successfully amplified) and the overall quantity of the extracted DNA. In an era of genomics and complete genomes, some groups are still far behind in the range of markers used to investigate their evolutionary history (e.g. Perkins et al. 2011; Littlewood et al. 2015; Chapter 3). This limitation will ultimately be reflected in the data available for taxonomy. The best way to improve the assessment of biodiversity is to add all sources of information, namely morphological. There are several difficulties associated with morphological approaches, especially when it comes to incorporating other kinds of evidence. Even when measurements are clearly described, errors associated with specimen preparation, imaging and individual variability between researchers, contributes to the difficulty of integrating those measurements in comparative studies. In our study, all previous morphological data was re-measured with the same imaging software to control for errors associated with software differences, as well as by the same person (FJ). International journals now commonly require authors to publish their data to free, publicly available, online databases (e.g GenBank; Benson et al. 2005). Morphological online databases such as MorphoBank (O'Leary et al. 2012) are also freely available. So similar to molecular data, morphological data from published studies should also be made available in such database. This way, comparative studies can benefit from the imaging data (i.e. voucher pictures) reducing the measuring error in comparative studies. Even under the term “integrative taxonomy”, there is a great variability in the methods used (Yeates et al. 2011). But prior to the selection of a unifying methodology for each group, an informative selection of characters should be made.

Conclusion

The use of an integrative framework for taxonomy does seem promising. This integrative approach will not only benefit taxonomy, but also generate data that can be incorporated into systematic studies. Nevertheless, incongruence between different sources of data may arise. Prior to conducting these analyses it is important to assess the reliability of morphological traits in recovering organism relatedness. In this study, we delimited *Spauligodon* parasitic nematode lineages based on molecular data and evaluated if morphological character divergence followed phylogenetic divergence. While traits evolved in a similar way, they do have a different structure. Understanding how morphological traits evolve and if they reflect the phylogenetic relatedness of groups will help to determine a proper set of morphological traits to be use in species

discrimination. Also, such knowledge will provide insights to the processes underlying the generation of morphological diversity.

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Supporting Information

Additional Supporting Information can be found in the Appendix E.

Data E.1: Descriptive statistics for all the linear measurements of adult specimens from the different *Spauligodon* lineages included in this study.

CHAPTER 7

General Discussion

Spauligodon atlanticus, adult female

Parasites are definitely not what it was expected them to be. The complexity of their interactions, suits better the diversity of organisms that adopted such a successful lifestyle. This thesis presents an integrative evolutionary study of the associations and evolution of *Spauligodon* parasitic nematodes. This thesis aimed to understand the processes driving parasite associations and diversification, how those processes are balanced, and if they influence parasite evolutionary rates and morphological diversification. The results obtained in previous chapters endorse the view of parasites as complex organisms, reflected in the parasite paradox of the dynamic of host use, morphological complexity (i.e. alternative male morphotypes), as well as the observed diversifying morphology as opposed to morphological stasis.

How host parasite interactions are established and evolve? A main question in the study of the evolutionary history of host-parasite associations is to determine how do they come to be. This was addressed in this study using parasitic nematodes belonging to the genus *Spauligodon* occurring in an island system, the Canarian archipelago. Phylogenetic evidence suggests that the *Spauligodon* nematodes from the Canary Islands evolved from at least four different lineages, but probably representing only three independent colonisation events (Chapter 2). The four parasitic lineages demonstrate a high degree of host specificity (measured as the number of host species used to complete their life cycle), even when observing the high host population densities that occur in sympatry. The degree of specificity (only one host species used per parasite lineage per island) did not prevent the parasite from switching to unrelated hosts, illustrating the parasite paradox in host use. Diversification of host use represented the main source of parasite diversification, providing the raw material for ensuing speciation processes. Island colonisation represented a geographic expansion and an oscillation period in the evolutionary history of *Spauligodon* parasites. The resulted mosaic structure of *Spauligodon* diversity and host associations in the Canary Islands can be explained by a combination of not “missing the boat” and association by descent. New host-parasite associations are further explained by host-switching in early stages of post-colonisation on the basis of ecological fitting, later followed by association by descent boosted by host specificity (Chapter 2).

Which is the balance between host specificity and ecological fitting? Ecological fitting promoted an initial niche enlargement and ultimately a niche shift. *Spauligodon* parasites may just use their host as a resource, tracking the resource provided by the host rather than host phylogeny. If so, then why do we observe such a degree of host specificity? If we assume that the observed host specificity is a result of dispersion and transmissions limitations, high host densities should promote more encounters between parasite infective stages and the host. Parasite life-history strategies with a direct life cycle, as seen in *Spauligodon* nematodes, should also facilitate

such encounters. It remains very intriguing that the parasite host switched when host densities were lower and encounter filters were narrower (*sensu* Combes 2001), but it does not do it now that host densities are higher and encounter filters are wider. Not all host switches result in range expansion. Currently, “occasional” host switches still occur, but each lineage still restricts the host use to a unique host species (Chapter 2). How can we explain it? Space and time are to be considered. Both space (ecological space) and time (evolutionary rate) are important drivers of both large-scale and small-scale species richness patterns by influencing diversification rates (Wiens 2011; Gillespie 2015). Host-parasite systems are not an exception, since they result from geographical, ecological and host-evolutionary associations on a temporal and spatial continuum (Hoberg and Brooks 2010). Cyclical episodes of expansion and isolation in geographic range, referred to as taxon pulses (Erwin 1985; Halas et al. 2005) are pointed as drivers of biotic structure and diversification (Halas et al. 2005; Hoberg and Brooks 2008; 2010; Hoberg et al. 2012). At a fine scale, individual host-parasite associations are also a result from interactions between ecological fitting and species traits along a temporal and spatial continuum.

Oscillations (geographic expansion, colonisations) foster cycles of change in ecological space enlarging the encounter filters. Ecological fitting provides the possibility for range expansion by host switching, which promotes variability in host use. Parasite life cycle strategies, fecundity and dispersion abilities will affect the outcome: range expansion or specialisation followed by possible codivergence. While time alone cannot explain diversity (species diversity or diversity in host-parasite association), time is required for evolutionary process to take place and produce outcomes (Wiens 2011; Gillespie 2015). Patterns of origin and evolution of host-parasite associations are generally not a result of a single influence, but rather the outcome of complex interactions along the space and time continuum. Space as a measure of ecological similarity seems to have an important influence in specialisation (Poulin 2005) and even infracommunity structure (Martin et al. 2005; Roca et al. 2005; Carretero et al. 2006; 2014). For most periods of times, host and parasite community structure will be stable enough for the development of host-specificity.

Taxa differ fundamentally in rates of evolutionary response and adaptive divergence after environmental changes (Gillespie 2015). While *Spauligodon* parasites present a high degree of specificity, it will be interesting to test the same dynamic model in other parasites presenting different degree of specificity and different life cycles. Oxyuridian species are expected to be highly specific (Adamson 1990). However, the degree of specificity does vary within members of the same family as it have been observed between *Spauligodon* and *Parapharyngodon*, with *Spauligodon* presenting a higher degree of specificity (Falk and Perkins 2013; de Sousa 2015). Both parasites present the same life cycle strategy, and are found in the same hosts (Roca et al. 2005; 2009; Falk and Perkins 2013; de Sousa 2015), but other genera specific traits account for

specificity differences, which in turn may be enough to explain differences in population structure and diversification (Nadler et al. 1995; Falk and Perkins 2013). Understanding host-parasite dynamics between the dichotomy of ecological fitting and host specificity is important to predict emergence of diseases, outcomes of introduced species and climate or habitat instability. Such knowledge is fundamental to understanding how complex resource users diversify. Parasites are very resilient, specialisation does not restrict their evolution neither lead to dead ends. Such flexibility in host use is one of the main contributors for parasite success as a mode of life.

The frequency of successful host switches in the evolutionary history of *Spauligodon* parasitic nematode (Chapter 2) leads to the question of what are the evolutionary consequences for parasites of different coevolutionary events. In particular, how does codivergence versus host shift determine their associations in the first place. *Will it alter the rate of molecular evolution?* Parasites are expected to undergo rapid evolution (Haraguchi and Sasaki 1996; Bromham et al. 2013). However, it is unclear if such rapid evolution is a direct consequence of their lifestyle or if other factors associated with their lifestyle (i.e. sharp and frequent bottlenecks) are responsible for such increase in rates (Bromham et al. 2013). Associated with their parasitic lifestyle are the dynamics of host-parasite associations. Understanding the effects of extreme coevolutionary events and host-switching versus codivergence could shed light on the complex nature of host-parasite associations. This new approach did not reach any conclusive results (Chapter 3). No significant differences in rates between topological congruent and incongruent parasite lineages, neither an influence of the degree of incongruence between topologies or time in parasite evolutionary rate was found. If rates did change it would imply that the dynamic of codivergence versus host switching would have direct consequences at the genome level. Host shifts followed by host specificity are considered one of the main drives in the diversification of *Spauligodon* parasites (Chapter 2). Host shifts have direct consequences on demography (i.e. sharp bottleneck) but may or may not affect antagonistic gene-by-gene interactions. Rates of molecular evolution are often correlated positively with diversification (Webster et al. 2003; Lancaster 2010; Bromham et al. 2015). Clarifying the links between genomic change (mutation) and species responses at microevolutionary scale (population divergence) and macroevolutionary scale (lineage diversification) may shed light on the dynamic processes of species diversification such as host-parasite evolutionary dynamics (Bromham et al. 2015). Nevertheless, it remains unclear to what extent bouts of rapid genetic evolution could arise from extreme genetic drift during bottlenecks or from adaptive pressures (Webster et al. 2005). Uncovering if rates of molecular evolution are influenced by evolutionary events such as host shifts is one more piece in the puzzle in the understanding of parasite diversification as consequence of historical associations.

After concentrating on parasite diversity of associations how they originated, diversify and constraint parasite molecular evolution, the focus of this thesis shifted to morphological diversity. Morphology is a separate but not independent component of organismal diversity. In this thesis the study of morphological diversity was performed under a combined approach of integrating molecular and morphological data. *Is there limited morphological diversity in parasites?* Contrary to the general assumption, there is no positive correlation between adopting a parasitic lifestyle and having reduced morphological diversity (Chapter 1). In the studied parasitic organism it was found that some lineages actually present two alternative male morphotypes (Chapter 4) and that morphological traits present a disparity in this parasitic nematode (Chapter 6), as opposed to unchanging morphology.

What drives the existence of alternative male phenotypes? The understanding of the evolutionary processes driving alternative phenotypes is still far from complete (Brockmann 2001; Oliveira et al., 2008). Extreme phenotypic diversity with two forms (two adaptive peaks) for one of the sexes, as seen in *Spauligodon* males, has been reported in other parasitic taxa (i.e. Nematoda, Oxyurida: Ainsworth 1990; Rhabditida: Hoberg et al. 2012). For *Spauligodon* parasitic nematodes male dimorphism was interpreted as a result of alternative reproductive tactic (Chapter 4). However, such interpretation remains dependent on further studies. Whereas morphotypes are a result from alternative adaptation, in the broad sense (West-Eberhard 1986), or alternative reproductive tactics (Gross 1996; Tomkins and Hazel 2007; Oliveira et al. 2008), this result demonstrates that parasites are still constrained by similar adaptive pressures as nonparasitic organisms. Adapting to a parasitic lifestyle does not restrict the type of evolutionary pressures to mere survival in the host. Parasites are also targets of selection from several different fronts. From taxonomic perspective, the identification of morphotypes highlights the importance of integrating not only morphological characters into the analyses, but also other data sources, particularly genetic data.

In addition to the extreme case of morphological variability with the presence of alternative phenotypes for the same sex, the presence of cryptic species (i.e. morphologically indistinguishable but genetically distinct species) was also investigated. Following the initial expectations that parasites are degenerative organisms, with limited morphological characters, the direct consequence would be the observation of several cryptic species within a particular parasitic taxa. The current ubiquitous use of molecular tools in parasitology has led to the discovery of numerous cryptic species, supporting this expectation (Nadler and Pérez-Ponce de León 2011; Poulin 2011). But, as Pérez-Ponce de León and Nadler (2010) highlighted, such definition of cryptic species should always be considered provisionally cryptic since additional morphological

studies or new high-resolution microscopy techniques may unveil diagnostic structural differences. *Is there cryptic diversity?* In the studied case of *S. occidentalis* and *S. atlanticus* the answered was negative (Chapter 5). The combination of morphometrics and SEM allowed the detection of phenotypic differences between the two species. Additionally, Chapter 6 provided further evidence that several male morphological characters present disparity that have accumulated linearly through time. However, it is premature to interpret this study as a generalisation that there is no morphological cryptic diversity in *Spauligodon* parasites. Nevertheless, it highlights the importance of understanding how characters evolve during the evolutionary history of an organism. Cryptic diversity is documented in several taxa, not only in parasitic ones (e.g. Adams et al. 2014). What is important is that a proper assessment of the nature of cryptic diversity is always performed. When cryptic nature of the species is confirmed additional studies will provide insides for the understanding of how morphological characters evolve, and what are the evolutionary conditions that drive the occurrence of cryptic diversity. Is it a convergent evolution or morphological stasis?

Can morphology be used to discriminate between parasite lineages? To answer this question a proper assessment of the morphological evolution of the taxon of interest is required. It is no longer proper to limit such morphological assessment to numerical analysis, since it fails to establish credible primary homologies (Ragsdale and Baldwin 2010). Different characters may be constrained by different evolutionary pressures (e.g. Kaliontzopoulou et al. 2015; Klaczko et al. 2015). Unravelling the diversification of morphological structures will have a positive contribution in taxonomic studies (i.e. identification of proper diagnostic traits) and to understand and resolve possible incongruences in integrative studies between morphological data and other source of evidence (i.e. molecular). In *Spauligodon* parasitic nematodes, all the analysed male morphological traits likely diverged under a Brownian model of evolution (Chapter 6). However, their phylogenetic signal varied between characters (Chapter 6). Some characters presented a strong phylogenetic signal, evolving dependently from the phylogeny, indicating their suitability for taxonomic procedures. Other characters presented a weak phylogenetic signal (weak dependence from the phylogeny of the observed trait assuming a pure Brownian model of evolution) which may limit, (but not prohibit) their use for *Spauligodon* lineage discrimination. It remains to be determined if female morphological characters evolve similarly, besides the general assumption that they have limited taxonomic value. Additionally, following the general assumption of convergent evolution in parasitic taxa, it would be interesting to determine if phylogenetic relatedness between hosts (as an estimation of similar habitat) could lead to convergent evolution. Preliminary results on male morphological characters point to morphological disparity that has been accumulated linearly through time under neutral drift of character change with no selection (Chapter 6). If morphological disparity in parasites follows their phylogenetic divergence, rather than host phylogenetic

relatedness it will represent further evidence that these parasites use their host as resources and are not contained by host selective pressures even though they can still specialise.

While several other important questions regarding parasite evolution were not addressed in this thesis, the studies presented here highlights the complex nature of parasitic organisms. Host-parasite associations are a result of several processes along a temporal and spatial continuum, as it occurs for non-parasitic taxa. Oscillations promote opportunity in space, ecological fitting the possibility, and time and species characteristics the resulting diversification. The degree to which such dynamics affect parasite evolutionary rates and morphology is still to be determined. *Spauligodon* parasitic nematodes demonstrate diversity in several areas, including host use, morphology, and rates of molecular change. Hopefully the contribution of this thesis highlights the possibilities and value of parasites as evolutionary models. Parasites are an integrative part of communities, how they evolve and diversify should be of interest of all evolutionary biologists, not only parasitologists.

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APPENDIX **A**

What you get is what they have? Detectability of intestinal parasites in reptiles using faeces

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Abstract

Parasitological analyses are often based on invasive methodologies, involving host sacrifice, raising ethical and conservation issues. However, alternative non-invasive approaches may not be always applicable due to the location of the parasite in the host tissue or the quality and reliability of the non-invasive sample *per se*. In this study, we compare the differences in detectability of intestinal parasites in reptiles using the classical invasive approach (intestine dissection), versus a non-invasive procedure (faecal examination), collected from the same individual host. Our results showed significantly lower detectability of helminths in faeces versus the intestine. Moreover, the number of parasites found in faeces was not explained either by the intensities found in the respective intestine or by the host identity. Several factors may explain the lack of association between the two types of samples, but more importantly, our results highlight the randomness of the presence of parasites in faeces. Even if it is not recommended that comparative studies of either parasite abundance or parasite communities be conducted on the basis of faecal samples, there are other types of studies (i.e. genetic) that can be performed with this source of information, thus avoiding the sacrifice of the host. Due to their wide spectrum of life stages and localization in the host tissue, parasites are challenging candidates for non-invasive sampling and consequently, parasitological methodologies should be carefully selected according to the objective of the study.

Introduction

Parasite assessment represents one of the biggest challenges in the inventory of the overall species present on the planet. Due to their small size and the high variety and complexity of life cycles and corresponding life stages, detection and identification of parasites are not straightforward. Estimation of parasite abundances, through the measurement of prevalence (the percentage of infected hosts in a population) and intensity of infection (the mean number of parasites per infected hosts), can be used to determine some basal characteristics of parasite species even when accounting for temporal variation (Poulin 2007). However, such studies are still missing for the majority of species of no economic importance. One of the major impediments for parasitological studies is that the sacrifice of the host is usually required because of parasite location in host tissues. In this context, metazoan parasites may be separated into ectoparasites (such as ticks and mites) and endoparasites (i.e. nematodes and trematodes). While ectoparasites are found on the external surface of their host, hence, being more accessible for collection, endoparasites are found in practically all host internal organs, requiring the sacrifice of the host for their collection. Thus, due to ethical considerations, especially in the case of threatened and rare species or species with low population sizes, when possible, non-invasive methodologies are recommended for parasite assessment. Non-invasive sampling has been implemented for a variety

of purposes: from diet studies (Carretero et al. 2006) to genetic assessment of rare or threatened species (Busby et al. 2009; Torre et al. 2013) and also for parasite surveys (Wimmer et al. 2004; Richter et al. 2011). However, such alternative methods may not be applicable for all parasite species, being restricted to parasites whose life stages occur in host body locations that allow their direct collection, like skin or that are accessible indirectly, i.e. through faeces and blood.

Due to the effects of parasites on host behaviour and fitness, which may impact community structure, they are increasingly becoming an important aspect to be considered in ecological studies (Poulin 1999; Preston and Johnson 2012). In this context, non-invasive sampling may be particularly advantageous since it does not interfere or jeopardize the structure of host communities, as sampling involving sacrifice would. Faeces from vertebrates have already been used for detection of gastrointestinal parasites such as coccidians (Couch et al. 1996; Daszak et al. 2011; Richter et al. 2011) and intestinal nematodes (Fenner et al. 2011; Jorge et al. 2011, 2012; Gyawali et al. 2013). Intestinal parasite communities have been traditionally assessed through direct intestinal analysis (i.e. Martin and Roca 2005; Diaz et al. 2013) but may also be accessed via non-invasive sampling through the identification of eggs, larvae and the adult forms that may be evacuated in the defaecation process (Couch et al. 1996; Millán et al. 2008; Acosta et al. 2011; Meijer et al. 2011; Zhang et al. 2011). However, intestinal parasites may be under-represented in the faeces due to their location in the intestine, their intensities of infection, presence of adult females shedding eggs and/or the erratic nature of faeces formation and release. Hence, the trade-off between the objectives of the study, the accuracy of the survey and the conservation status of the host must be considered carefully before selecting the appropriate sampling methodology. In this study, we compare the differences in detectability of intestinal parasites in reptile hosts between two types of samples, faecal pellets and the whole intestinal tract, collected from the same individual hosts, focusing on prevalence and abundance of infection. We then discuss the advantages and disadvantages of the invasive versus non-invasive approaches.

Material and Methods

Parasitological procedures

Fifty-two lizard specimens, from eleven species, were collected from different localities (Table A.1) between 2008 and 2012. For each individual, two types of samples, faecal pellets and intestines, were obtained. Faeces were collected first either through spontaneous defaecation of the reptiles when captured or by gentle abdominal massage. Intestinal samples were obtained from the same specimens, which died accidentally during fieldwork or were later taken to the laboratory and euthanized through inhalation of ether vapours, dissected and the intestine removed. Research

Table A.1 Prevalence, intensity and mean intensity of the total intestinal parasites (all species pooled) detected in the two types of samples (I: intestine, F: faeces) from each respective host species.

Host family	Host species	Sample type	N Samples	Infected	Prevalence (%)	Total Intensity	Mean Intensity
Lacertidae	*Gallotia atlantica mahoratae*	I	2	1	50	1	1
Lacertidae	*Gallotia atlantica mahoratae*	F	2	0	0	0	0
Lacertidae	*Gallotia caesaris caesaris*	I	2	2	100	149	74.5
Lacertidae	*Gallotia caesaris caesaris*	F	2	0	0	0	0
Lacertidae	*Gallotia caesaris gomerae*	I	12	11	92.7	407	37
Lacertidae	*Gallotia caesaris gomerae*	F	12	9	75	206	22.9
Lacertidae	*Gallotia galloti eisentrauti*	I	2	2	100	364	182
Lacertidae	*Gallotia galloti eisentrauti*	F	2	1	50	1	1
Lacertidae	*Gallotia galloti palmae*	I	2	2	100	104	52
Lacertidae	*Gallotia galloti palmae*	F	2	0	0	0	0
Lacertidae	*Gallotia stehlini*	I	1	1	100	10	10
Lacertidae	*Gallotia stehlini*	F	1	0	0	0	0
Lacertidae	*Atlantolacerta andreanszkyi*	I	1	0	0	0	0
Lacertidae	*Atlantolacerta andreanszkyi*	F	1	0	0	0	0
Lacertidae	*Podarcis carbonelli*	I	1	0	0	0	0
Lacertidae	*Podarcis carbonelli*	F	1	0	0	0	0
Lacertidae	*Podarcis hispanica*	I	1	0	0	0	0
Lacertidae	*Podarcis hispanica*	F	1	0	0	0	0
Lacertidae	*Podarcis hispanica* Type 2	I	12	12	100	273	22.75
Lacertidae	*Podarcis hispanica* Type 2	F	12	4	33.3	4	1
Lacertidae	*Podarcis sicula*	I	13	5	38.5	18	3.6
Lacertidae	*Podarcis sicula*	F	13	2	15.4	8	4
Lacertidae	*Podarcis vaucheri*	I	1	0	0	0	0
Lacertidae	*Podarcis vaucheri*	F	1	0	0	0	0
Phyllodactylidae	*Tarentola angustimentalis*	I	1	0	0	0	0
Phyllodactylidae	*Tarentola angustimentalis*	F	1	0	0	0	0
Phyllodactylidae	*Tarentola rudis rudis*	I	1	1	100	38	38
Phyllodactylidae	*Tarentola rudis rudis*	F	1	0	0	0	0
	Intestines samples Total		52	37	71,1	1364	36.9
	Faeces samples Total		52	16	30.8	219	13.7
	Total		104	53		1583	

[a] See Kaliontzopoulou et al. (2011) for information on *Podarcis* lineages.

protocols were approved by the responsible regional authorities [Cabildos Insulares licences: Lanzarote (no. 4889), Fuerteventura (no. 3298 and no. 12570), Gran Canaria (no. 10983), Tenerife (no. 358/ 2009), La Palma (no. 2009006659), La Gomera (no. 5145) and El Hierro (ref. CGO/rsh) from Spain; Servicio de Protección y Conservación de la Naturaleza, Dirección General del Medio Natural, Consejería de Desarrollo Sostenible y Ordenación del Territorio de la Región de Murcia (licence no. Sol/CPA/ASO/156-08) and Junta de Andalucía (licence no. SGB/FOA/AFR 2010, reg. 17461) from Spain; ICNB from Portugal (licence no. 69/2011/CAPT); Direcção Geral do Ambiente, MAA, from Cape Verde (licence no. 07/2008) and Haut Commissariat Eaux et forêts et à la lutte contre la Desertification from Morocco (licence no. 14/HCEFLCD/ DLCDPN/DPRN/CFF)]. Both types of samples were preserved in 96 % ethanol and further analysed using a stereo-microscope

in search of helminth parasites. The parasites were then separated, counted and identified to the generic level. Egg forms were not considered because they were few in numbers and are difficult to identify properly.

Statistical analyses

Presence analysis: To determine if detectability of intestinal parasites was similar between the two types of samples, faeces and intestines, McNemar's chi-squared test with continuity correction (function mcnemar.test of the *R* package) was applied on the 2×2 contingency table for the presence and/ or absence of parasites in matched pairs of intestine and faeces [I.e. total number of hosts where parasites were found in the intestine, but not in the faeces (I+/F−), total number of hosts where parasites were found in both samples types (I+/F+)]. Analyses were performed for all parasites and for the most common parasite genera (*Spauligodon*, *Thelandros* and *Parapharyngodon*). All analyses were performed using the package R version 2.15.1 (R Core Team Development 2011).

Intensity analysis: The relationship between the number of parasites found in the intestine and in the faeces from the same host individual was tested using a nonparametric Spearman correlation (function rcorr, R package *Hmisc*, Harrel et al. 2013). In order to determine which factors might explain the number of parasites, we performed a repeated analysis of variance (function ezANOVA, R package *ez*, Lawrence 2012) using number of parasites as dependent variable, type of sample as within-subjects factor and parasite genus or host species as between-subjects factors. Repeated analysis of variance was also performed for each of the three more common parasite genera (*Spauligodon*, *Thelandros* and *Parapharyngodon*), but using type of sample as within-subjects factor and host species as between-subject factor. Similarly, the same analysis was also performed for the most common hosts (*Gallotia caesaris gomerae*, *Podarcis sicula* and *Podarcis hispanica* PH2), this time using type of sample as within-subjects factor and parasite genus as between-subjects factor. All analyses were performed using the package R version 2.15.1 (R Core Team Development 2011).

Results

From the 52 lizards (lacertid lizards and geckos) analysed, 39 individuals were found infected with intestinal parasites (parasites found in the intestine and/or in the faeces). A total of 1,583 parasites from six different genera were detected from which 86 % were found in the intestines and 14 % in faeces. One parasite was identified as Cestoda, while all the others were nematodes of the family Pharyngodonidae (Oxyurida). *Spauligodon*, *Thelandros* and *Parapharyngodon* were the most common genera (46, 39 and 13 % of the total number of parasites, respectively), while

Tachygonetria (0.6 %) and *Skrjabinodon* (0.2 %) were less frequent. In addition, four Pharyngodonidae larvae (0.2 %) were found but they could not be assigned to any genus. Intensities and prevalences for each host taxon are detailed in Table A.1.

Our analysis showed strong discrepancies in the detection of parasites depending on the type of sample analysed (Fig.A.1). From the total of 39 specimens infected, 37 (95 % of cases) contained parasites in their intestine, but in only 14 of these specimens (36 %), parasites were also found in the respective faeces. This pattern was similar in separate analyses for each of the three most common parasite genera, i.e. *Spauligodon*, *Parapharyngodon* and *Thelandro*s (Fig.A.1 and A.2). In two host specimens, intestinal parasites were detected only in the faeces, with no parasite been detected in the corresponding intestine (total intensities: 2 and 15).

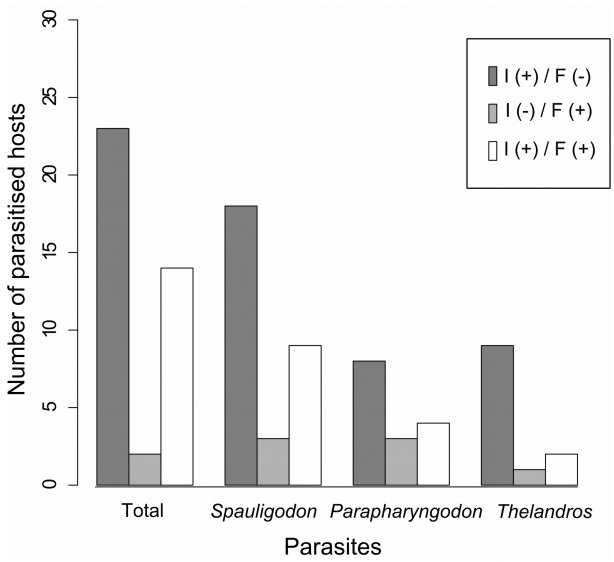

Fig.A.1- Bar plot representing the host frequency for the different levels of detectability of matched pairs of intestine and faeces samples for all the parasites species (pooled) and for the three most common parasites. Dark grey bars represent hosts with parasites in the intestine but not in the faeces [I(+)/F(−)], light grey bars include the number of hosts with parasites in faeces but not in the intestine [I(−)/F(+)] and white bars represent hosts with parasites observed both in the intestine and respective faeces [I(+)/F(+)].

There was significant discordance between the detectability of helminths in intestines and faeces, considering that the marginal probabilities of each type of sample should be the same (McNemar chi-squared=16, df =1, P <0.001). Differences in detectability were also significant when considering each nematode genus separately, namely, *Spauligodon* (McNemar's chi-squared=9.33, df =1, P <0.005), *Thelandros* (McNemar's chi-squared=4.9, df =1, P <0.05) but not for *Parapharyngodon* (McNemar's chi-squared=1.45, df =1, P = 0.2278; the other genera were not analysed due to the small sample size).

Regarding intensities, the number of parasites found in the intestines was higher than in the faecal samples (repeated measures ANOVA, within-subjects factor = type of sample, $F_{1,63}$ = 8.20, P =0.006) but the correlation between them was not significant (Spearman correlation, rho=−0.19, P =0.24). However, variation in the number of parasites did not depend either on the host species

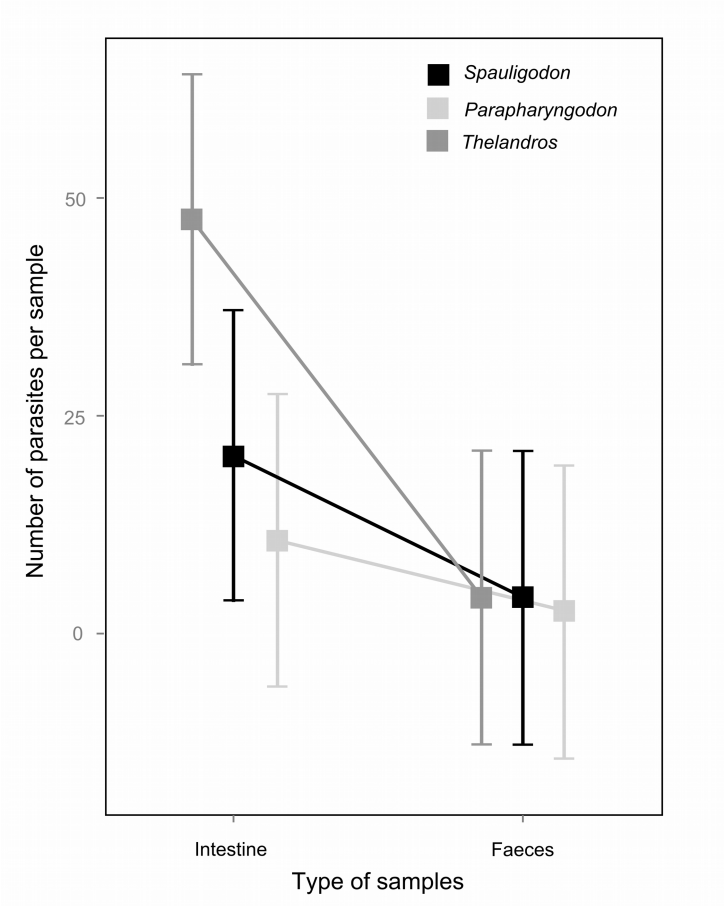

Fig.A.2- Differences in the abundance of the three main parasite groups between intestinal and faecal samples. For the three genera, mean (and standard error) number of parasites per sample are shown.

(repeated measures ANOVA, within-subjects factor=type of sample, $F_{1,55}$ =8.80, *P* = 0.004; between-subjects factor = host species, $F_{8,55}$ = 1.24, *P* = 0.292; interaction, F 8,55 = 1.57, *P* =0.156) or on parasite genus (within-subjects factor = type of sample, $F_{1,57}$ = 7.98, *P* = 0.006; between-subjects factor = parasite genus, $F_{6,57}$ = 0.95, *P* = 0.465; interaction, $F_{6,57}$ = 0.72, *P* = 0.635). Similar results were obtained when the three most common parasite genera were individually analysed. *Spauligodon* was more abundant in the intestine than in the faeces (repeated measures ANOVA, within-subjects factor = type of sample, $F_{1,24}$ = 11.85, *P* =0.002; between-subjects factor = host species, $F_{5,24}$ =1.58, *P* = 0.203; interaction, $F_{5,24}$ = 1.72, *P* = 0.169) and showed a negative, though not significant trend in the correlation of abundances between both types of samples (Spearman, rho = −0.32, *P* = 0.08). For *Parapharyngodon*, the number of parasites was similar in intestines and faeces and for host species (repeated measures ANOVA, within-subjects factor = type of sample, $F_{1,10}$ = 3.11, *P* = 0.108; between-subjects factor = host species, $F_{5,10}$ = 1.48, *P* = 0.280; interaction, $F_{5,10}$ = 0.79, *P* = 0.577), although again, a negative, not significant, correlation between sample types was detected (Spearman, rho = −0.28, *P* = 0.31). For *Thelandros*, we did find significant differences in all comparisons (repeated measures ANOVA,

within-subjects factor = type of sample, $F_{1,8}$ = 22.45, *P* = 0.0015; between-subjects factor = host species, $F_{3,8}$ = 49.67, *P* = 0.00002; interaction, $F_{3,8}$ = 37.13, *P* = 0.0005). Also in this case, no correlation between the numbers of parasites in intestines and faeces was detected (Spearman correlation, rho = −0.46, *P* = 0.13).

Within each of the most common host species (*G. caesaris gomerae*, *P. hispanica* PH2 and *P. sicula*), there were no differences between the number of parasites found in intestines and faeces and no effect of parasite genus on numbers of parasites per sample (in both species, repeated measures ANOVA, within-subjects factor = type of sample, *P* >0.05; between-subjects factor = parasite genus, *P* >0.05; interaction, *P* > 0.05), the only exception being *P. hispanica* PH2. For this host species, there was a significantly higher number of parasites in the intestines than in the faeces but no effect of the parasite genus (repeated measures ANOVA, within-subjects factor = type of sample, $F_{1,11}$ = 53.10, *P* = 0.00005; between-subjects factor = parasite genus, $F_{1,11}$ =4.22, *P* =0.064; interaction, $F_{1,11}$ = 4.86, *P* = 0.0496). No correlation between the number of parasites detected in both types of samples was observed in any of the three host species analysed (Spearman correlation, in all cases *P* >0.05).

Discussion

The majority of helminths found in both types of samples, faeces and intestines, were adult forms of nematodes belonging to the family Pharyngodonidae (Oxyurida), which are commonly found in the intestinal helminth communities of reptiles (i.e. Martin et al. 2005), with the genus *Spauligodon* being the most common. Pharyngodonidae nematodes inhabit the last part of the intestine of their host and they live free in it, while other helminths such as cestodes and trematodes are usually found in the upper part of the intestine fixed to the intestinal mucosa and thus are less likely to be dislodged in faeces. However, the detectability of Pharyngodonidae nematodes was significantly lower in faeces than in the intestine. Faeces were collected before the intestine content was analysed, and the helminths retrieved from the faeces were probably dislodged and evacuated when faeces were expelled. It has been reported that in oxyurids, gravid females may pass out of the host and function as oothecae (Adamson 1990), which may be an additional reason for the presence of adult nematode females in the faeces. Contrary to our expectation, no positive correlation was found between the helminth intensities found in the faeces and that in the paired intestine, i.e. a higher intensity of helminths in the intestine was not accompanied by a higher intensity of helminths in faeces. We only detected an effect of the type of sample (detecting a higher number of parasites in the intestine than in faeces), but no effect of the host or parasite taxonomic identities on the number of parasites found. The absence of correlation may be due to the spatial distribution of the parasites within the intestine and to the random

deposition of parasites in the forming faeces. The same pattern was uncovered in separate analyses for each of the three more common parasites. Depending on the total length of the host or their diet, the intestinal tract can vary in size and shape (i.e. length and presence of a caecum; Stevens and Hume 1995; Carretero 2004) which may influence the spatial distribution of parasites and consequently influence their probability of being dislodged during the defaecation process. Although *G. caesaris gomerae* presents a larger body size and a greater tendency to herbivory compared with *Podarcis* lizards (Roca 1999; Martin et al. 2005), no effect of host species was detected on the intensity of parasites found in faeces. The only exception was in the separate analysis for the nematode genus *Thelandros*, where a significant effect of host species was detected. However, this was probably due to the finding of a single host intestine containing 359 individuals of this parasite.

Considering our results regarding the lack of correlation between both types of samples, we conclude that comparative studies on parasite abundance or parasite community studies performed on the basis of faecal samples should be interpreted very cautiously. Similar results were obtained regarding diet studies performed on stomach versus intestine samples (Carretero and Llorente 2001), highlighting that different source of samples, even if *a priori* related, may not yield comparable information. The presence of nematode parasites or their eggs in faeces has been used to estimate parasite prevalence, intensities or abundances in several organisms, especially for those large mammals in which obtaining large sample sizes involving necropsy are not possible due to their endangered status or sampling difficulties (Ashford et al. 1996; Millán et al. 2008; Acosta et al. 2011; Zhang et al. 2011). Ecological studies based solely on faeces most probably underestimate the true abundance and diversity present at the community level and consequently will not reflect the real helminth community. Other methodologies of faeces examination, i.e. faecal floatation or molecular based techniques, could yield higher parasite detection. However, the same uncorrelated pattern may still be found regarding the deposition or ability of faeces to drag adult parasites or other parasite stages while forming or during defaecation.

Nevertheless, faeces are still a reasonable alternative for baseline surveys. The variety of studies that can be conducted on helminths retrieved from faeces will depend on the collection technique. Fresh samples, collected directly and stored in ethanol, will allow both morphological (even given some degree of shrinkage) and genetic analyses to be conducted. Based on our results and the typical low prevalence of reptile intestinal helminths, we recommend that a large number of faecal samples should be collected to increase the likelihood of detection of intestinal parasites. Similarly, it can also be important to aim at temporal replication, which would allow greater insight into the true parasite fauna present in a given host population and/or locality. Another advantage of using faecal samples is that parasite surveys using this method do not alter

the population structure of the host, such that repeated sampling of the same host individual (i.e. for monitoring seasonal variation) is possible. Nevertheless, preliminary studies may be first performed in order to assess the reliability of non-invasive sampling for each group of parasite and host. In fact, the best methodology would be combining the two types of samples, i.e. faeces that may be expelled during the capture process and the intestinal content, since, as seen in some specimens, faeces sometimes may provide the best representation of an infracommunity. Several studies have also been conducted on museum specimens of hosts (i.e. Hartigan et al. 2010; Bursey and Goldberg 2012). However, these types of samples are usually not viable for genetic analysis due to the preservation medium since helminths are located in tissues that are more prone to degradation. In conclusion, parasitological methodologies should be carefully selected according to the objective of the study. However, different parasites with different life stages and with different prevalence and intensities in a given host will inevitably present different sampling challenges. Unfortunately, because of the internal location of most parasites, non-invasive sampling approaches are not always possible and the sacrifice of the host will often be required for accurate estimates of abundance or diversity.

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APPENDIX **B**

Supporting information for Chapter 2

Data B.1. Nematode specimens used in the phylogenetic analyses of Chapter 2, including their respective host species, locality and GenBank accession numbers.

Species	Code	Host	Locality	GenBank 18S rDNA	GenBank 28S rDNA	GenBank COI	Reference
S. occidentalis (Canarian clade A1)	S2447F	*G. c. caesaris*	El Hierro Spain	JF829235	JF829261	JF829306	Jorge et al. 2011
S. occidentalis (Canarian clade A1)	S19454MA	*G. c. caesaris*	El Hierro, Spain	-	-	KJ778107	Jorge et al. 2014
S. occidentalis (Canarian clade A1)	S19454MI	*G. c. caesaris*	El Hierro, Spain	KJ778076	KJ778097	KJ778106	Jorge et al. 2014
S. occidentalis (Canarian clade A1)	S2450F1	*G. c. caesaris*	El Hierro, Spain	-	JF829260	JF829308	Jorge et al. 2011
S. occidentalis (Canarian clade A1)	S2458F	*G. c. caesaris*	El Hierro, Spain	-	-	JF829313	Jorge et al. 2011
S. occidentalis (Canarian clade A1)	S2467F	*G. c. caesaris*	El Hierro, Spain	-	JF829258	JF829303	Jorge et al. 2011
S. occidentalis (Canarian clade A1)	S16492F[I]	*G. atlantica*	Fuerteventura, Spain	x	x	x	This study
S. occidentalis (Canarian clade A1)	S23057[I]	*G. atlantica*	Fuerteventura, Spain	-	x	-	This study
S. occidentalis (Canarian clade A1)	S2091MA	*G. c. gomerae*	La Gomera, Spain	-	-	JF829294	Jorge et al. 2011
S. occidentalis (Canarian clade A1)	S2490F	*G. c. gomerae*	La Gomera, Spain	-	-	JF829314	Jorge et al. 2011
S. occidentalis (Canarian clade A1)	S2504F	*G. c. gomerae*	La Gomera, Spain	-	-	JF829297	Jorge et al. 2011
S. occidentalis (Canarian clade A1)	S2525F	*G. c. gomerae*	La Gomera, Spain	-	-	JF829299	Jorge et al. 2011
S. occidentalis (Canarian clade A1)	S2525MA	*G. c. gomerae*	La Gomera, Spain	-	-	JF829300	Jorge et al. 2011
S. occidentalis (Canarian clade A1)	S19330MA	*G. g. palmae*	La Palma, Spain	x	x	x	This study
S. occidentalis (Canarian clade A1)	S19336F	*G. g. palmae*	La Palma, Spain		x	x	This study
S. occidentalis (Canarian clade A1)	S2088F	*G. g. palmae*	La Palma, Spain	JF829233	JF829259	JF829293	Jorge et al. 2011
S. occidentalis (Canarian clade A1)	S1277F	*G. galloti*	Tenerife, Spain	-	-	JF829289	Jorge et al. 2011
S. occidentalis (Canarian clade A1)	S19253MA	*G. galloti*	Tenerife, Spain	x	x	x	This study
S. occidentalis (Canarian clade A1)	S2089F	*G. galloti*	Tenerife, Spain	-	-	JF829290	Jorge et al. 2011
S. occidentalis (Canarian clade A1)	S2095F	*G. galloti*	Tenerife, Spain	-	-	JF829311	Jorge et al. 2011
S. occidentalis (Canarian clade A1)	S2123	*G. galloti*	Tenerife, Spain	JF829231	-	-	Jorge et al. 2011
S. occidentalis (Canarian clade A1)	S2124F	*G. galloti*	Tenerife, Spain	-	JF829256	JF829292	Jorge et al. 2011
S. occidentalis (Canarian clade A1)	S23235F	*G. galloti*	Tenerife, Spain	-	x	x	This study
S. occidentalis (Canarian clade A1)	S19278F[I]	*T. delalandii*	Tenerife, Spain	x	x	x	This study
Canarian clade A2	S19453F	*C. coeruleopunctatus*	El Hierro, Spain	-	-	x	This study
Canarian clade A2	S19471F	*C. coeruleopunctatus*	El Hierro, Spain	x	x	x	This study
Canarian clade A2	S19478MA	*C. coeruleopunctatus*	El Hierro, Spain	-	x	x	This study
Canarian clade A2	S23224F	*C. coeruleopunctatus*	El Hierro, Spain	x	x	x	This study
Canarian clade A2	S19416MA[I]	*G. c. caesaris*	El Hierro, Spain	x	-	x	This study
Canarian clade A2	SGccV2MA	*G. c. caesaris*	El Hierro, Spain	-	-	x	This study
Canarian clade A2	S19352MA	*C. coeruleopunctatus*	La Gomera, Spain	x	x	x	This study
Canarian clade A2	S23142F3	*C. viridanus*	Tenerife, Spain	x	-	x	This study
Canarian clade A2	S23142F2	*C. viridanus*	Tenerife, Spain	-	x	x	This study
Canarian clade A2	S23142MIx	*C. viridanus*	Tenerife, Spain	-	x	x	This study
S. atlanticus (Canarian clade B)	S1337F2	*G. atlantica*	Fuerteventura, Spain	-	JF829249	JF829282	This study
S. atlanticus (Canarian clade B)	S1338F	*G. atlantica*	Fuerteventura, Spain	-	-	JF829284	Jorge et al. 2011
S. atlanticus (Canarian clade B)	S1367F	*G. atlantica*	Fuerteventura, Spain	-	-	JF829280	Jorge et al. 2011
S. atlanticus (Canarian clade B)	S1368F	*G. atlantica*	Fuerteventura, Spain	JF829230	JF829251	JF829285	This study
S. atlanticus (Canarian clade B)	S16536MI	*G. atlantica*	Fuerteventura, Spain	KJ778075	KJ778099	KJ778108	Jorge et al. 2014
S. atlanticus (Canarian clade B)	S23239F	*G. atlantica*	Gran Canaria, Spain	x	x	x	This study
S. atlanticus (Canarian clade B)	S1339F	*G. atlantica*	Lanzarote, Spain	-	-	JF829275	Jorge et al. 2011
S. atlanticus (Canarian clade B)	S1341F	*G. atlantica*	Lanzarote, Spain	-	-	JF829272	Jorge et al. 2011
S. atlanticus (Canarian clade B)	S1383	*G. atlantica*	Lanzarote, Spain	JF829232	-	JF829273	Jorge et al. 2011
S. atlanticus (Canarian clade B)	S1407F	*G. atlantica*	Lanzarote, Spain	-	-	JF829277	Jorge et al. 2011

Data B.1. (cont.)

Species	Code	Host	Locality	GenBank 18S rDNA	GenBank 28S rDNA	GenBank COI	Reference
S. atlanticus (Canarian clade B)	S1441F	*G. atlantica*	Lanzarote, Spain	-	-	JF829278	Jorge et al. 2011
S. atlanticus (Canarian clade B)	S1492F	*G. atlantica*	Lanzarote, Spain	-	JF829250	JF829274	Jorge et al. 2011
S. atlanticus (Canarian clade B)	S1492F2	*G. atlantica*	Lanzarote, Spain	-	-	JF829279	Jorge et al. 2011
S. atlanticus (Canarian clade B)	S16108F	*G. atlantica*	Lanzarote, Spain	x	x	x	This study
S. atlanticus (Canarian clade B)	S16154F	*G. atlantica*	Lanzarote, Spain	-	x	x	This study
S. atlanticus (Canarian clade B)	S15142*	*T. angustimentalis*	Lanzarote, Spain	x	x	-	This study
Canarian clade C	S23199MA	*C. coeruleopunctatus*	El Hierro, Spain	x	-	-	This study
Canarian clade C	SL2H3F	*C. coeruleopunctatus*	El Hierro, Spain	x	x	x	This study
Canarian clade C	S19418F	*G. c. caesaris*	El Hierro, Spain	x	-	x	This study
Canarian clade D	S23080F2!	*C. sexlineatus*	Gran Canaria, Spain	x	-	-	This study
Canarian clade D	S23069	*T. b. boettgeri*	Gran Canaria, Spain	-	x	-	This study
Canarian clade D	S23071F	*T. b. boettgeri*	Gran Canaria, Spain	x	x	x	This study
Canarian clade D	S23168F4	*T. gomerensis*	La Gomera, Spain	x	-	x	This study
S. aloisei	S7158F2	*P. sicula*	Menorca, Spain	x	x	x	This study
S. auziensis	S14717MA	*T. mauritanica*	Morocco	x	x	x	This study
S. auziensis	STF4F	*T. mauritanica*	Morocco	x	x	x	This study
S. cabrerae	SAP1F	*P. lilfordi*	Addaia Petit, Spain	-	x	x	This study
S. cabrerae	S24287F	*P. lilfordi*	Dragonera, Spain	x	x	x	This study
S. carbonelli	SmpenF	*P. hispanica* PH1A	Portugal	KJ778080	KJ778090	x	Jorge et al. 2014
S. carbonelli	S13432MA	*P. hispanica* PH2	Portugal	KJ778082	KJ778092	KJ778111	Jorge et al. 2014
S. extenuatus	S23854F	*Ti. tangitanus*	Morocco	x	x	x	This study
S. extenuatus	S9553F	*Ps. algirus*	Morocoo	x	x	x	This study
S. extenuatus	S20400MA	*Ti. lepidus*	Portugal	x	x	x	This study
S. lacertae	SLm28F	*L. media*	Armenia	JF829237	JF829255	JF829287	Jorge et al. 2011
S. lacertae	S10057F	*L. strigata*	Armenia	JF829238	JF829252	JF829286	Jorge et al. 2011
S. nicolauensis	S2597F	*T. bocagei*	São Nicolau, Cape Verde	JF829226	JF829243	JF829265	Jorge et al. 2011
S. nicolauensis	S2828F	*T. nicolauensis*	São Nicolau, Cape Verde	KJ778087[a]	JN619358[b]	JN619359[b]	[a] Jorge et al. 2014 / [b] Jorge et al. 2012
S. saxicolae	S9902F	*D. unisexualis*	Armenia	JF829227	JF829246	JF829266	Jorge et al. 2011
S. saxicolae	SDr12EF	*D. rudis*	Turkey	x	x	x	This study
Spauligodon sp.	SJCB6417MA	*T. ephippiata*	Mauritania	x	x	x	This study
Spauligodon sp.	S14064F	*P. muralis*	Montseny, Spain	x	x	x	This study
Spauligodon sp.	S23963MA	*C. ocellatus*	Morocco	x	-	-	This study
Spauligodon sp.	S3320F	*Chalcides. sp.*	Morocco	-	x	x	This study
Spauligodon sp.	S1757MA	*P. vaucheri*	Morocco	x	x	x	This study
Spauligodon sp.	S9211MA	*P. vaucheri*	Morocco	JF829228	JF829247	JF829269	Jorge et al. 2011
Spauligodon sp.	S14167F	*T. desertii*	Morocco	x	x	x	This study
Spauligodon sp.	S14187F	*T. desertii*	Morocco	x	x	x	This study
Spauligodon sp.	SG4F	*T. gigas*	Raso, Cape Verde	KJ778088	KJ778096	KJ778105	Jorge et al. 2014
Spauligodon sp.	SG9MA	*T. gigas*	Raso, Cape Verde	KJ778086	KJ778095	KJ778104	Jorge et al. 2014
Spauligodon sp.	S15043MA	*C. ocellatus*	Sardinia, Italy	x	x	x	This study
Spauligodon sp.	S15120MA	*P. tiliguerta*	Sardinia, Italy	KJ778079	KJ778100	KJ778109	Jorge et al. 2014
Spauligodon sp.	S15448MA	*P. tiliguerta*	Sardinia, Italy	x	x	x	This study
T. tinerfensis	T19408M	*T. gomerensis*	La Gomera, Spain	KJ778073	KJ778089	x*	Jorge et al. 2014
P. echinatus	P1345F	*G. atlantica*	Fuerteventura, Spain	JF829224	JF829241	JF829263	Jorge et al. 2011
P. echinatus	P1328F	*G. atlantica*	Fuerteventuraa, Spain	JF829223	JF829240	JF829262	Jorge et al. 2011

Gallotia (G), Gallotia galloti (G. g.), Gallotia caesaris (G. c.), Tarentola (T.), Tarentola boettgeri (T. b.), Chalcides (C.), Podarcis (P.), Timon (Ti), Psammodromus (Ps.), Lacerta (L.), Darevskia (D.); ! Host-switch event; x, unsubmitted sequence data to GenBank*;*

** Reference: This study.*

Data B.2. Reptile specimens used in the cophylogenetic analysis of Chapter 2, including their locality and GenBank accession numbers.

Code	Species	Locality	GenBank 12S rDNA	Reference
DB2447	*G. c. caesaris*	El Hierro Spain	x	This study
DB19471	*C. coeruleopunctatus*	El Hierro, Spain	x	This study
DB19478	*C. coeruleopunctatus*	El Hierro, Spain	x	This study
DB23224	*C. coeruleopunctatus*	El Hierro, Spain	x	This study
DB19454	*G. c. caesaris*	El Hierro, Spain	x	This study
DB2458	*G. c. caesaris*	El Hierro, Spain	x	This study
DB2464	*G. c. caesaris*	El Hierro, Spain	x	This study
DB16492	*G. atlantica*	Fuerteventura, Spain	x	This study
DB19352	*C. coeruleopunctatus*	La Gomera, Spain	x	This study
DB19330	*G. g. palmae*	La Palma, Spain	x	This study
DB19336	*G. g. palmae*	La Palma, Spain	x	This study
DB2088	*G. g. palmae*	La Palma, Spain	x	This study
DB23142	*C. viridanus*	Tenerife, Spain	x	This study
DB19253	*G. galloti*	Tenerife, Spain	x	This study
DB2124	*G. galloti*	Tenerife, Spain	x	This study
DB23235	*G. galloti*	Tenerife, Spain	x	This study
DB19278	*T. delalandii*	Tenerife, Spain	x	This study

Gallotia (*G*), *Gallotia galloti* (*G. g.*), *Gallotia caesaris* (*G. c.*), *Tarentola* (*T.*), *Chalcides* (*C.*). x, unsubmitted sequence data to GenBank.

Data B.3. Saturation plots for the COI third codon positions.

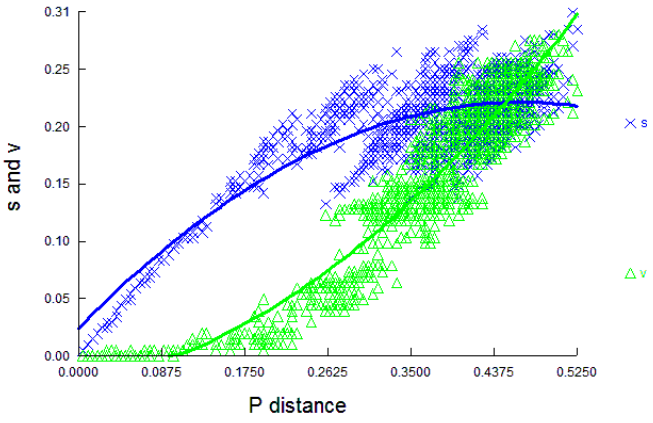

Fig.B.1- Observed number of transitions (s) and transversions (v) plotted against uncorrected distances for the COI third codon positions.

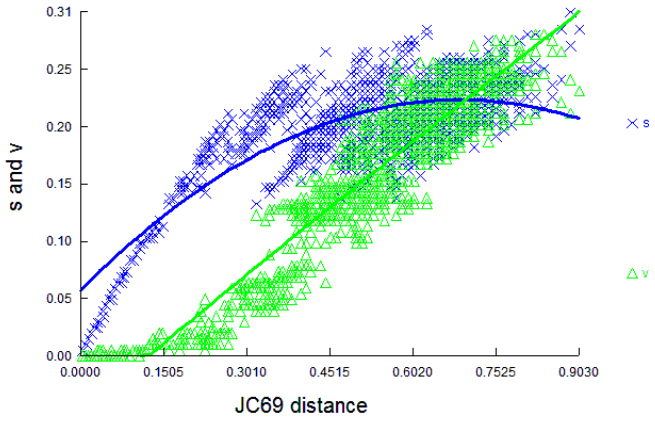

Fig.B.2- Observed number of transitions (s) and transversions (v) plotted against and JC69 distances for the COI third codon positions.

APPENDIX **C**

Supporting information for Chapter 4

Data C.1. Number of samples collected in Chapter 4 study from each host species. (I: intestine; F: faeces).

Family	Species	Locality	Sample Type	N Samples
Lacertidae	*Darevskia bendimahiensis*	Turkey	I	5
Lacertidae	*Darevskia clarkorum*	Turkey	I	29
Lacertidae	*Darevskia rudis*	Turkey	I	308
Lacertidae	*Darevskia unisexualis*	Turkey	I	11
Lacertidae	Darevskia uzzelli	Turkey	I	20
Lacertidae	*Darevskia valentini*	Turkey	I	24
Lacertidae	*Gallotia atlantica atlantica*	Lanzarote Island, Spain	F	45
	Gallotia atlantica atlantica	Lanzarote Island, Spain	I	24
Lacertidae	*Gallotia atlantica laurae*	Lanzarote Island, Spain	F	23
	Gallotia atlantica laurae	Lanzarote Island, Spain	I	10
Lacertidae	*Gallotia atlantica mahoratae*	Fuerteventura Island, Spain	F	36
	Gallotia atlantica mahoratae	Fuerteventura Island, Spain	I	30
Lacertidae	*Gallotia caesaris caesaris*	E El Hierro Island, Spain	F	47
	Gallotia caesaris caesaris	E El Hierro Island, Spain	I	20
Lacertidae	*Gallotia caesaris gomerensis*	La Gomera Island, Spain	F	20
	Gallotia caesaris gomerensis	La Gomera Island, Spain	I	20
Lacertidae	*Gallotia galloti eisentrauti*	Tenerife Island, Spain	F	2
	Gallotia galloti eisentrauti	Tenerife Island, Spain	I	20
Lacertidae	*Gallotia galloti galloti*	Tenerife Island, Spain	F	10
	Gallotia galloti galloti	Tenerife Island, Spain	I	20
Lacertidae	*Gallotia galloti palmae*	La Palma Island, Spain	F	28
	Gallotia galloti palmae	La Palma Island, Spain	I	30
Lacertidae	*Podarcis hispanica* PH2	Portugal	F	19
	Podarcis hispanica PH2	Portugal	I	20
Lacertidae	*Podarcis tiliguerta*	Sardinia Island, Italy	F	83
	Podarcis tiliguerta	Sardinia Island, Italy	I	2
Phyllodactylidae	*Tarentola gigas*	Raso Island, Cape Verde	F	10
		Intestines samples total		593
		Faecal samples total		323
		Total		916

APPENDIX **D**

Supporting information for Chapter 5

Data D.1. Descriptive statistics for all the linear measurements of adult specimens from the different localities included in Chapter 5 (in µm). For each variable mean ± standard deviation (SD), range, and sample size (*N*) is given.

	Eastern Lineage							
	Nazaret - Lanzarote				Lajares - Fuerteventura			
	Males (*N*=9)		Females (*N*=9)		Males (*N*=9)		Females (*N*=9)	
Character	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range
BL	898.47 ± 107.04	697.73-1075.1	3256.75 ± 318.72	2934.49-3796.86	788.55 ± 108.82	659.4-939.84	2436.14 ± 361.14	1835.28-2818.8
BW	125.2 ± 20.14	94.58-160.04	412.66 ± 39.59	351.92-473.09	147.18 ± 16.91	116.44-170.48	323.82 ± 41.87	273.35-408.85
OL	153.94 ± 19.35	130.18-183.34	271.73 ± 15.33	254.03-297.76	143.28 ± 18.22	106.04-165.66	251.41 ± 19.08	222.32-290.53
OW	24.48 ± 2.58	20.01-27.5	36.49 ± 3.74	29.24-41.76	22.43 ± 2.21	18.47-25.47	31.32 ± 3.12	25.1-34.93
OBL	55.82 ± 6.92	47.55-68.51	89.56 ± 3.71	85.32-96.11	52.35 ± 4.82	42.93-56.47	77.97 ± 8.07	63.42-88.64
OBW	60.85 ± 5.4	49.78-66.76	101.89 ± 5.83	94.21-111.46	56.99 ± 5.01	46.36-64.1	91.98 ± 4.87	84.14-96.67
NR	70.99 ± 17.77	40.42-99.17	111.24 ± 17.21	87.55-150	80.15 ± 9.75	65.69-94.12	103.67 ± 12.71	80.97-122.4
ExP	263.86 ± 33.07	228.07-328.44	242.49 ± 63.31	183.48-367.84	241.41 ± 27.72	205.2-297.99	228.19 ± 78.53	132.67-374.99
TL	242.19 ± 22.57	199.72-278.29	493.24 ± 35.21	430.08-539.26	194.8 ± 60.1	110.17-264.74	468.94 ± 21.93	451.35-505.22
LA	52.23 ± 14.56	39.44-68.39	-		54.41 ± 9.87	45.22-65.87	-	
EC1	19.8 ± 1.7	17.64-22.25	-		20.47 ± 1.4	18.75-22.8	-	
EC2	12.12 ± 1.32	10.35-13.82	-		12.13 ± 1.27	9.25-13.78	-	
TW	13.45 ± 1.01	12.06-14.9	-		12.24 ± 1.33	9.34-14.11	-	
3p1	5.39 ± 0.82	3.85-6.71	-		5.4 ± 0.46	4.42-5.92	-	
3p2	4.2 ± 0.79	3.23-5.61	-		3.81 ± 0.49	3.01-4.54	-	
3p3	9.45 ± 0.74	8.36-10.38	-		7.9 ± 1.07	6.52-9.52	-	
3pl	8.81 ± 1.11	7.48-10.88	-		7.85 ± 0.95	6.12-9.53	-	
Vu	-		289.8 ± 62.16	231.76-408.13	-		275.32 ± 85.5	162.13-413.76
Va	-		474.81 ± 66.05	396.91-591.01	-		421.48 ± 74.13	303.33-521.79
Weggm	-		35.77 ± 4.17	28.01-47.60	-		36.51 ± 3.60	32.07-45.38
Leggm	-		122.04 ± 5.01	103.94-133.33	-		120.57 ± 5.98	104.98-131.32
Spines	0		8 ± 0.93	6-9	0		6.67 ± 0.58	6-7

	Western Lineage							
	Valverde - El Hierro				E. N. Sra. Reyes - El Hierro			
	Males (*N*=9)		Females (*N*=9)		Males (*N*=9)		Females (*N*=9)	
Character	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range
BL	1528.81 ± 187.17	1263.79-1749.14	3626.55 ± 536.83	3006.88-4457.37	1451.18 ± 83.13	1334.89-1595.01	3383.33 ± 325.29	3040.81-3950.47
BW	193.71 ± 10.65	174.14-204.29	438.18 ± 52.26	369.94-516.24	175.18 ± 25.42	140.99-219.02	372.89 ± 39.99	305.75-411.8
OL	271.06 ± 25.41	231.33-316.67	392.22 ± 31.71	325.1-426.59	285.95 ± 22.1	249.78-316.87	378.43 ± 21.04	333.93-400.03
OW	27.6 ± 3.55	22.63-32.83	37.78 ± 2.68	32.01-40.88	26.87 ± 3.04	22.41-31.83	37.88 ± 3.79	32.28-44.32
OBL	71.58 ± 9.99	59.35-90.1	104.33 ± 6.62	91.37-112.39	72.48 ± 8.6	61-85.07	101.94 ± 6.38	94.27-110.14
OBW	79.55 ± 9.09	68.15-98.21	118.24 ± 12.21	99.56-141.33	77.84 ± 8.46	67.29-94.51	112.6 ± 11.52	92.96-125.92
NR	113.79 ± 22.01	70.57-142.68	123.85 ± 12.37	104.56-142.25	128.33 ± 21.2	98.23-153.73	125.57 ± 7.76	111.84-134.77
ExP	432.73 ± 69.8	325.59-557.85	289.48 ± 82.27	203.52-405.08	417.05 ± 23.35	379.79-456.47	353.35 ± 55.34	236.05-417.03
TL	141.78 ± 7.03	134.21-155	398.67 ± 54.14	331.34-510.33	130.32 ± 14.57	114.59-148.34	405.53 ± 14.49	388.88-415.25
LA	95.89 ± 26.89	62.51-125.01	-		-	-	-	
EC1	31.81 ± 3.36	26.31-35.71	-		30.76 ± 2.49	27.96-35.49	-	
EC2	16.74 ± 3.52	12.02-22.14	-		16.58 ± 1.44	14.45-19.28	-	
TW	12.14 ± 1.1	10.37-14.06	-		12.46 ± 1.07	10.94-14.21	-	
3p1	8.62 ± 1.21	7.47-11.03	-		8.79 ± 0.92	7.32-9.89	-	
3p2	5.78 ± 0.56	5.07-6.83	-		5.56 ± 0.85	3.88-6.63	-	
3p3	9.67 ± 1.37	8.3-12.55	-		9.85 ± 0.78	8.67-11.34	-	
3pl	11.65 ± 1.56	10.14-14.55	-		13.76 ± 0.68	12.85-14.9	-	
Vu	-		357.49 ± 91.73	239.28-475.61	-		411.37 ± 57.97	273.13-465.23
Va	-		569.01 ± 83.06	463.4-727.45	-		532.37 ± 60.62	452.96-647.85
Weggm	-		37.45 ± 2.52	33.71-42.37	-		40.58 ± 2.83	36.00-47.63
Leggm	-		132.02 ± 4.80	123.38-139.34	-		130.49 ± 4.49	118.96-139.43
Spines	0		6.78 ± 1.2	5-9	0		7.17 ± 1.17	6-9

Data D.1. (cont.)

	Western Lineage					
	Las Casetas - La Gomera				La Lomada - La Palma	
	Males (*N*=9)		Females (*N*=9)		Males (*N*=9)	
Character	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range
BL	1139.02 ± 113.98	983.75-1298.43	2769.04 ± 278.33	2323.64-3160.69	1406.83 ± 136.39	1217.32-1631.86
BW	153.23 ± 19.23	124.97-177.21	366.05 ± 40.28	295.02-412.19	137.88 ± 25.59	98.93-184.25
OL	265.91 ± 9.62	253.14-286.92	403.13 ± 27.08	356.41-449.3	260.36 ± 21.83	229.19-301.23
OW	24.4 ± 2.2	20.13-27.41	32.6 ± 1.94	29.59-36.77	25.99 ± 3.14	20.44-29.7
OBL	64.16 ± 7.74	51.2-74.98	100.3 ± 8.71	87.16-113.49	69.87 ± 6.69	60.06-79.35
OBW	70.86 ± 9.02	55.43-81.29	117.35 ± 6.45	110.86-128.75	78.89 ± 9.22	63.56-91.7
NR	125.9 ± 10.89	109.49-148.88	123.48 ± 10.14	110.24-142.97	124.55 ± 10.63	107.21-144.05
ExP	382.82 ± 48.63	328.47-480.26	292.52 ± 53.97	227.26-382.16	406.57 ± 18.42	371.44-439.69
TL	137.64 ± 18.78	100.47-165.66	407.99 ± 52.39	350.56-505.9	109.69 ± 8.54	98.95-120.83
LA	129.44 ± NA	129.44-129.44	-		55.07 ± NA	55.07-55.07
EC1	29.97 ± 2.51	27.05-33.34	-		32.17 ± 2.13	29.24-35.36
EC2	15.05 ± 0.78	13.94-16.5	-		19.36 ± 2.79	16.22-24.42
TW	12.75 ± 1.78	10.21-15.3	-		12.36 ± 1.38	10.67-15.27
3p1	8.55 ± 1.32	6.69-10.7	-		8.64 ± 0.65	7.69-9.56
3p2	5.03 ± 1.86	2.22-8.06	-		6.41 ± 0.9	4.79-7.39
3p3	9.18 ± 1.11	7.65-11.01	-		10.55 ± 1.56	8.06-13.23
3pl	11.46 ± 1.18	10.1-13.17	-		11.91 ± 1.69	9.14-14.95
Vu	-		334.34 ± 55.05	270.37-438.43	-	
Va	-		516.9 ± 68.58	437.32-636.34	-	
Weggm	-		41.13 ± 2.58	37.24-46.43	-	
Leggm	-		139.19 ± 4.49	131.78-152.27	-	
Spines	0		7.75 ± 1.28	6-10	0	

	Western Lineage			
	San Miguel - Tenerife			
	Males (*N*=9)		Females (*N*=9)	
Character	Mean ± SD	Range	Mean ± SD	Range
BL	1061.42 ± 159.32	915.56-1446.99	2689.42 ± 259.8	2343.9-3202.04
BW	142.53 ± 16.07	113.72-174.18	353.3 ± 38.8	309.88-435
OL	236.92 ± 20.28	202.06-273.06	379.95 ± 27.89	342.93-424.26
OW	24.24 ± 2.06	22.14-28.01	35.48 ± 2.4	31.59-39.33
OBL	62.36 ± 9.87	49.61-85.19	105.59 ± 5.98	96.06-115.73
OBW	69.58 ± 12.06	51.96-92.61	119.99 ± 6.02	107.03-127.01
NR	106.18 ± 11.34	90.21-121.18	122.21 ± 10.72	104.01-137.85
ExP	339.06 ± 40.66	288.07-392.8	273.56 ± 52.2	200.08-377.13
TL	115.89 ± 5.89	106.54-124.79	357.17 ± 64.9	267.97-463.57
LA	88.46 ± 8.49	82.46-94.46	-	
EC1	28.25 ± 2.4	23.83-32.51	-	
EC2	15.13 ± 2.46	11.9-18.39	-	
TW	11.91 ± 0.92	10.38-12.92	-	
3p1	7.8 ± 1.23	5.25-9.36	-	
3p2	4.63 ± 1.01	2.81-6.09	-	
3p3	9.51 ± 0.8	7.63-10.26	-	
3pl	11.52 ± 1.32	10.37-13.97	-	
Vu	-		339.02 ± 49.75	294.09-447.29
Va	-		508.49 ± 75.5	390.63-599.97
Weggm	-		41.38 ± 4.29	35.84-51.45
Leggm	-		127.30 ± 5.13	115.97-139.05
Spines	0		7.56 ± 1.59	6-11

APPENDIX **E**

Supporting information for Chapter 6

Data E.1. Descriptive statistics for all the linear measurements of adult specimens from the different lineages included in Chapter 6 (in µm). For each variable mean, standard deviation (sd), minimum (min) and maximum (max) values, and sample size (N) is given.

	Spauligodon Type A (N = 8)				*Spauligodon cabrerae* (N = 3)			
Character	mean	sd	min	max	mean	sd	min	max
BL	851.040	258.683	127.905	538.173	924.893	332.096	561.684	1213.020
X1pW	7.785	1.305	1.162	5.537	9.982	2.933	7.049	12.915
X2pW	8.824	2.581	1.703	5.503	15.287	2.574	12.713	17.861
X2pl	15.364	2.274	1.810	11.182	24.200	0.388	23.812	24.588
CT2	15.581	3.653	0.910	12.868	15.632	1.023	14.609	16.655
X3p1	6.187	0.936	1.053	4.646	7.780	0.751	7.030	8.531
X3pl	9.453	0.961	0.627	8.112	12.409	0.203	12.207	12.612
PextL	62.791	9.889	10.235	43.669	69.188	21.758	44.185	83.821
PextW1	39.646	6.035	5.838	28.742	46.811	1.861	44.950	48.672
X3pW	29.464	1.475	1.635	26.743	38.507	11.119	19.406	39.929
GenC	21.278	4.108	4.250	16.796	26.698	1.249	25.450	27.947
EBL	42.607	5.777	1.969	30.911	41.275	3.228	37.845	44.252
EBW	49.950	11.663	11.749	38.718	40.699	5.716	34.142	44.625

	Spauligodon Type D (N = 3)				*Spauligodon atlanticus* (N = 10)			
Character	mean	sd	min	max	mean	sd	min	max
BL	858.591	322.836	515.783	1156.821	848.606	97.955	705.013	1006.585
X1pW	7.024	0.839	6.398	7.978	8.095	1.726	6.491	11.482
X2pW	7.451	2.012	5.263	9.221	10.043	2.662	4.866	13.622
X2pl	13.508	5.180	9.611	19.387	15.357	3.248	9.023	19.352
CT2	15.158	0.485	14.814	15.713	12.968	1.404	10.518	14.795
X3p1	6.045	1.133	4.910	7.175	5.533	0.416	4.901	6.199
X3pl	8.352	2.456	5.525	9.963	8.106	1.028	7.178	10.382
PextL	64.719	3.844	60.281	67.006	56.164	4.819	48.942	63.989
PextW1	32.507	5.084	28.464	38.215	35.860	3.142	30.342	40.970
X3pW	28.810	3.709	25.444	32.786	27.417	2.655	23.389	32.423
GenC	20.658	2.723	17.514	22.284	22.043	2.577	17.786	26.417
EBL	43.107	2.179	41.242	45.502	63.871	13.116	45.875	81.871
EBW	44.532	9.150	34.614	52.645	65.757	15.597	39.925	90.575

	Spauligodon occidentalis (N = 10)				*Spauligodon* Type CanA2 (N = 10)			
Character	mean	sd	min	max	mean	sd	min	max
BL	1467.081	197.686	1171.473	1720.833	957.671	206.541	712.802	1453.206
X1pW	11.689	1.308	10.056	14.382	7.945	0.861	6.418	9.174
X2pW	14.099	2.804	9.694	18.969	11.047	1.581	7.648	12.610
X2pl	23.031	3.733	17.544	28.841	16.808	4.047	12.358	24.165
CT2	18.119	2.559	13.208	23.298	15.113	1.458	13.062	17.778
X3p1	9.019	1.153	7.480	11.525	6.825	0.738	5.927	8.360
X3pl	14.902	2.996	12.555	22.711	10.622	1.032	8.939	12.136
PextL	59.464	9.486	40.398	75.468	63.487	9.330	48.565	80.588
PextW1	49.589	13.966	11.123	59.637	51.009	8.434	41.419	71.258
X3pW	41.217	4.786	35.567	50.026	32.117	2.371	28.135	35.749
GenC	25.229	2.565	22.033	29.834	22.060	2.912	17.797	27.588
EBL	60.925	11.083	42.657	74.171	60.411	14.247	34.339	85.559
EBW	62.941	14.507	35.727	81.383	64.802	19.530	26.753	104.317

Data E.1. (cont.)

	Spauligodon extenuatus (N = 3)				*Spauligodon lacertae* (N = 2)			
Character	mean	sd	min	max	mean	sd	min	max
BL	1579.396	179.475	1375.995	1715.481	1756.252	12.544	1747.382	1765.122
X1pW	12.771	1.100	11.824	13.977	8.650	2.640	6.783	10.516
X2pW	20.767	3.854	17.354	24.947	21.717	2.067	20.255	23.178
X2pl	28.374	4.335	24.836	33.210	21.090	1.030	20.362	21.818
CT2	22.729	2.002	20.418	23.928	17.508	0.812	16.933	18.082
X3p1	9.611	0.617	8.899	9.983	7.368	0.386	7.095	7.641
X3pl	18.835	1.139	17.543	19.694	14.429	0.288	14.225	14.632
PextL	82.146	24.899	55.740	105.198	105.570	0.197	105.431	105.709
PextW1	70.000	1.957	67.928	71.818	81.017	6.167	76.656	85.377
X3pW	54.970	4.365	50.753	59.470	43.992	2.592	42.159	45.825
GenC	31.226	2.443	28.816	33.700	28.267	3.964	25.464	31.070
EBL	65.734	3.571	61.688	68.443	41.197	0.000	41.197	41.197
EBW	70.775	5.908	66.435	77.503	39.928	0.000	39.928	39.928

	Spauligodon saxicolae Type TK (N = 1)	*Spauligodon saxicolae* Type Ir (N = 2)				*Spauligodon* Type O (N = 4)			
Character	mean	mean	sd	min	max	mean	sd	min	max
BL	1172.551	832.510	56.510	792.551	872.468	1266.842	155.147	1131.583	1489.884
X1pW	11.466	8.294	0.018	8.281	8.307	10.079	1.266	9.109	11.867
X2pW	12.569	6.859	1.720	5.643	8.075	19.096	4.606	12.266	21.923
X2pl	22.831	10.668	2.844	8.657	12.679	18.972	1.336	17.162	20.311
CT2	14.105	14.500	0.772	13.954	15.046	17.020	4.290	10.720	19.999
X3p1	6.341	6.207	0.276	6.011	6.402	7.752	1.870	5.559	10.106
X3pl	8.313	7.246	0.064	7.200	7.291	10.658	1.324	9.481	12.553
PextL	73.819	68.142	3.969	65.335	70.948	70.228	5.035	64.958	75.929
PextW1	41.358	36.827	2.396	35.133	38.521	51.109	4.312	44.811	54.468
X3pW	29.024	23.268	0.450	22.949	23.586	33.449	3.017	29.249	35.982
GenC	27.795	18.274	0.000	18.274	18.274	24.013	3.643	19.552	28.475
EBL	39.975	79.283	2.739	77.346	81.220	59.943	2.845	57.064	63.674
EBW	41.318	88.089	0.044	88.058	88.120	73.090	6.628	66.617	79.926

	Spauligodon carbonelli (N = 8)				*Spauligodon auziensis* Type1 (N = 4)			
Character	mean	sd	min	max	mean	sd	min	max
BL	841.530	276.441	495.257	1217.740	897.056	307.050	608.501	1289.859
X1pW	9.367	2.177	7.646	13.559	8.333	2.600	5.150	11.497
X2pW	8.870	2.267	6.490	13.015	10.152	3.574	5.534	14.083
X2pl	11.726	2.016	8.738	15.507	15.887	4.410	10.075	20.129
CT2	11.168	2.304	7.385	14.260	13.561	1.519	11.494	15.102
X3p1	5.923	0.945	4.453	6.900	6.178	1.329	4.396	7.560
X3pl	7.583	2.265	5.524	11.657	9.871	3.577	6.394	13.819
PextL	61.678	8.938	44.840	72.780	52.825	9.276	44.611	65.582
PextW1	33.592	4.446	27.894	41.491	40.524	11.823	29.873	54.601
X3pW	25.645	4.437	19.973	32.569	32.365	5.579	26.957	37.652
GenC	19.276	3.869	14.429	27.417	23.472	6.777	16.502	29.510
EBL	55.270	8.502	41.789	72.336	70.138	14.213	49.346	81.458
EBW	54.215	12.828	34.519	78.869	80.728	13.846	60.134	90.085

Data E.1. (cont.)

	Spauligodon auziensis Type2 (N = 2)				*Spauligodon* Type V (N = 2)			
Character	mean	sd	min	max	mean	sd	min	max
BL	1249.616	470.010	917.268	1581.963	1351.909	40.228	1323.463	1380.354
X1pW	13.268	1.063	12.516	14.020	9.520	0.787	8.963	10.076
X2pW	21.858	6.496	17.264	26.451	29.124	4.320	26.069	32.179
X2pl	21.997	1.565	20.890	23.103	24.649	3.923	21.875	27.423
CT2	12.366	0.000	12.366	12.366	14.658	0.419	14.361	14.954
X3p1	10.710	2.391	9.019	12.401	8.852	0.000	8.852	8.852
X3pl	14.906	4.213	11.927	17.885	16.223	0.000	16.223	16.223
PextL	57.619	4.204	54.646	60.591	74.323	0.885	73.697	74.949
PextW1	70.174	16.079	58.804	81.543	72.286	10.595	64.794	79.778
X3pW	40.345	4.957	36.840	43.850	44.367	0.656	43.903	44.831
GenC	28.135	3.533	25.636	30.633	31.335	0.455	31.013	31.656
EBL	77.591	0.000	77.591	77.591	44.178	10.349	36.860	51.495
EBW	85.563	0.000	85.563	85.563	45.389	20.157	31.136	59.642

	Spauligodon paratectipenis (N = 2)				*Spauligodon* Type AB (N = 3)			
Character	mean	sd	min	max	mean	sd	min	max
BL	737.131	86.389	676.045	798.217	1308.891	285.294	982.731	1512.062
X1pW	6.752	0.138	6.654	6.849	12.884	2.399	10.279	15.001
X2pW	9.752	0.115	9.671	9.833	11.541	1.606	10.200	13.321
X2pl	13.810	0.996	13.106	14.514	25.071	2.358	22.400	26.863
CT2	14.327	5.754	10.258	18.396	9.444	2.328	6.867	11.395
X3p1	5.676	0.303	5.462	5.890	6.683	1.298	5.865	8.180
X3pl	8.654	0.896	8.020	9.287	15.536	1.493	14.375	17.220
PextL	48.003	2.090	46.525	49.480	40.253	7.385	31.778	45.313
PextW1	32.335	5.837	28.207	36.462	54.455	6.090	47.568	59.127
X3pW	28.674	2.234	27.094	30.253	37.421	1.933	35.442	39.304
GenC	18.904	0.327	18.672	19.135	16.748	2.761	13.890	19.401
EBL	46.741	4.074	43.860	49.621	65.716	8.537	55.915	71.525
EBW	54.722	13.205	45.385	64.059	70.188	5.626	63.701	73.729

	Spauligodon Type CanD1 (N = 4)				*Spauligodon* Type CanD2 (N = 1)	*Spauligodon* Type AC (N = 2)			
Character	mean	sd	min	max		mean	sd	min	max
BL	889.830	223.210	572.735	1057.438	1104.154	864.905	204.842	720.060	1009.750
X1pW	7.657	1.739	5.776	9.987	11.830	5.692	0.635	5.243	6.141
X2pW	8.059	1.612	5.660	9.043	10.318	7.338	0.855	6.733	7.942
X2pl	23.758	13.165	14.238	43.242	23.065	13.327	0.503	12.971	13.682
CT2	5.016	1.568	3.232	6.512	5.237	6.582	0.000	6.582	6.582
X3p1	5.202	0.930	4.008	6.145	5.866	4.753	0.046	4.720	4.785
X3pl	11.686	1.408	10.396	13.577	11.763	8.151	1.286	7.242	9.060
PextL	32.631	7.936	23.658	40.547	35.746	29.615	5.078	26.024	33.205
PextW1	36.339	4.941	31.505	43.242	44.684	35.727	1.798	34.455	36.998
X3pW	28.449	2.656	25.638	31.336	29.734	23.187	2.860	21.165	25.209
GenC	15.203	0.589	14.535	15.969	25.037	13.726	0.000	13.726	13.726
EBL	62.245	9.634	51.752	71.740	34.913	49.792	0.000	49.792	49.792
EBW	65.790	12.596	52.858	76.623	34.096	36.265	0.000	36.265	36.265

Data E.1. (cont.)

	Spauligodon nicolauensis (N = 5)			*Spauligodon* Type U (N = 3)			
Character	mean	sd	min	mean	sd	min	max
BL	1022.647	158.993	834.181	1110.795	169.305	963.881	1295.950
X1pW	5.621	0.446	4.986	6.963	1.391	5.369	7.931
X2pW	6.243	0.507	5.745	8.757	0.919	7.699	9.360
X2pl	15.167	1.685	12.563	15.064	1.627	13.186	16.040
CT2	6.151	0.000	6.151	8.418	0.000	8.418	8.418
X3p1	4.725	0.606	4.118	6.003	0.242	5.726	6.171
X3pl	8.338	1.391	6.758	10.111	1.102	9.010	11.213
PextL	25.019	2.804	20.305	33.749	0.263	33.486	34.012
PextW1	27.488	1.564	25.913	36.712	2.706	33.765	39.085
X3pW	19.847	1.329	18.155	25.122	2.018	23.955	27.452
GenC	17.289	1.718	15.341	20.351	0.141	20.266	20.514
EBL	58.540	21.284	29.809	51.565	4.289	46.668	54.651
EBW	56.659	24.735	29.103	54.617	8.802	46.044	63.631

Lineage K (N = 4)				Lineage Q (N = 3)			
mean	sd	min	max	mean	sd	min	max
1266.842	155.147	1131.583	1489.884	1308.891	285.294	982.731	1512.062
10.079	1.266	9.109	11.867	12.884	2.399	10.279	15.001
19.096	4.606	12.266	21.923	11.541	1.606	10.200	13.321
18.972	1.336	17.162	20.311	25.071	2.358	22.400	26.863
17.020	4.290	10.720	19.999	9.444	2.328	6.867	11.395
7.752	1.870	5.559	10.106	6.683	1.298	5.865	8.180
10.658	1.324	9.481	12.553	15.536	1.493	14.375	17.220
70.228	5.035	64.958	75.929	40.253	7.385	31.778	45.313
51.109	4.312	44.811	54.468	54.455	6.090	47.568	59.127
33.449	3.017	29.249	35.982	37.421	1.933	35.442	39.304
24.013	3.643	19.552	28.475	16.748	2.761	13.890	19.401
59.943	2.845	57.064	63.674	65.716	8.537	55.915	71.525
73.090	6.628	66.617	79.926	70.188	5.626	63.701	73.729